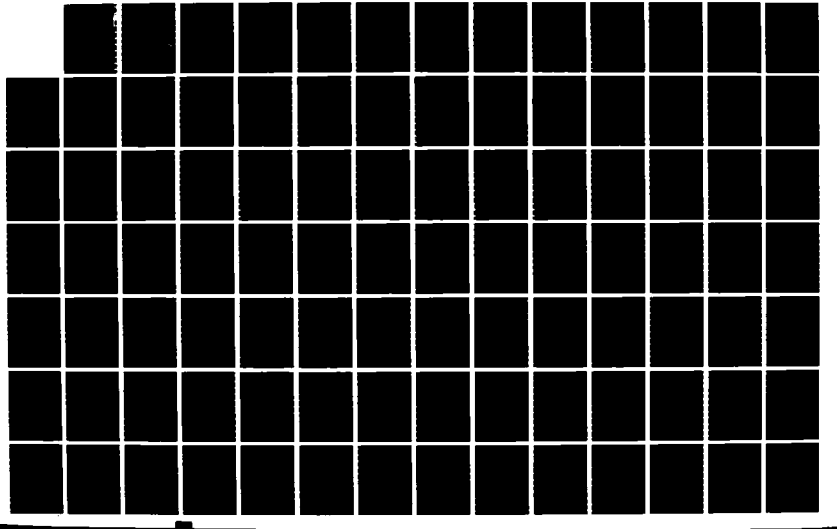
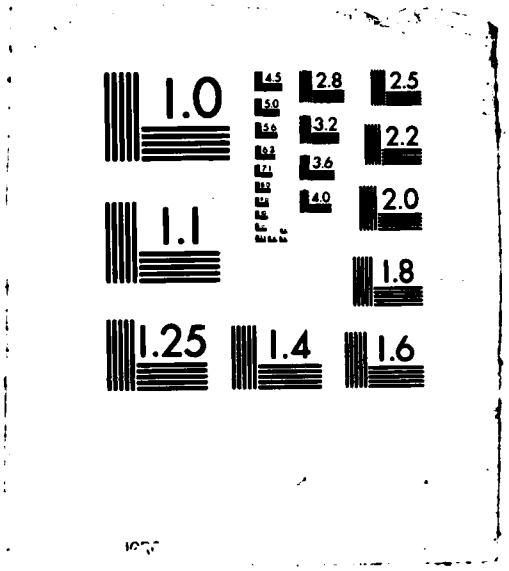
AD-A172 119 ACUTE NECROTIZING ULCERATIVE GINGIVITIS: MICROBIAL AND IMMUNOLOGIC STUDIES(U) MARYLAND UNIV BALTIMORE DENTAL SCHOOL W A FALKLER 05 AUG 84 DAMD17-80-C-0181 1/2

UNCLASSIFIED F/G 6/5 NL




1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6
6.3

ACUTE NECROTIZING ULCERATIVE GINGIVITIS: MICROBIAL AND IMMUNOLOGIC STUDIES

Annual/Final

(1/15/81-1/14/84)

William A. Falkler, Jr., Ph.D.

Supported by

U. S. Army Medical Research and Development Command

Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD-17-80-C-0181

University of Maryland Dental School

Baltimore, Maryland 21201

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO. / 3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle): Acute Necrotizing Ulcerative Gingivitis: Microbial and Immunologic Studies	5. TYPE OF REPORT & PERIOD COVERED: Annual/Final 1/15/81 - 1/14/84
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): Dr. William A. Falkler, Jr.	8. CONTRACT OR GRANT NUMBER(s): DAMD17-80-0181
9. PERFORMING ORGANIZATION NAME AND ADDRESS: University of Maryland Dental School, Baltimore, Maryland 21201	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS: SGRD-RMS, Technical Review Group, U.S. Army Medical Research and Development Command, Fort Detrick, Md. 21701	12. REPORT DATE: 8/5/84
	13. NUMBER OF PAGES: 145
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report)
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release;
distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

DOD Distribution Statement B (Proprietary Info.)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Periodontal Disease	Spirochetes
Microbiology	Fusobacterium
Immunology	Bacteroides

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This is the final report on a research project aimed at obtaining new information as to the microbial etiology and immunopathology of acute necrotizing ulcerative gingivitis (ANUG). Thirty five patients have been studied; 54% were male and 46% female, 18 were caucasion and 17 were black. The mean age of the patients was 23 years with a range of 14 to 50 years. The patients upon entering the clinic felt that they were healthy with the chief complaint being "sore gums". Clinical observations of the patients included cratering of papilla, fetid odor, pseudomembrane formation, blunting of papilla and lymphadenopathy. / Subgingival plaque samples taken from the patients revealed the

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

presence of large numbers of spirochetes and Gram (-) rods. Cultural studies showed that the Gram (-) rods comprised the highest percentage (78%) of the total organisms isolated. Of this group Bacteroides gingivalis and Fusobacterium nucleatum were the most frequently isolated. / Studies were undertaken to determine possible colonization and pathogenic mechanisms of B. gingivalis and F. nucleatum. Experiments were performed to determine whether hemolysis would occur following attachment to RBCs of strains of F. nucleatum and Bacteroides species including B. gingivalis. The F. nucleatum strains displayed both hemagglutination and hemolytic activity. The B. gingivalis strains and other Bacteroides species displayed hemagglutination but no measurable hemolytic activity. The hemolytic moiety was observed in various cell, cell wall and lipopolysaccharide extracts. / F. nucleatum, F. periodonticum and F. nucleatum isolates from individuals with chronic periodontitis, juvenile periodontitis, ANUG and healthy gingiva were compared serologically to each other and to other Fusobacterium species and F. nucleatum isolated from canines and a monkey. Antigen preparations of the human isolates, typed strains, F. periodonticum and two of the monkey strains shared lines of identity in immunodiffusion testing, none of the other Fusobacterium species reacted. As observed in immunodiffusion testing, all human isolates of F. nucleatum, the typed strains and F. periodonticum shared antigens when reacted with rabbit anti-F. nucleatum sera in immunoelectrophoresis. An ELISA in which rabbit anti-F. nucleatum and human sera were used revealed that the human and monkey isolates when used as antigens, allowed the detection of similar degrees of antibody activity; however, no activity was observed with the canine isolates. None of the canine isolates displayed the characteristic hemagglutination of sheep erythrocytes demonstrated by the human and monkey strains. / Antisera produced reactive with Bacteroides fragilis, B. gingivalis, or B. melaninogenicus subspecies melaninogenicus were used in radial immunodiffusion testing to examine the serological reactivity of antigen preparations of 43 strains of various species of Bacteroidaceae. Anti-B. gingivalis serum exhibited strong reactions with the strains of homologous species and reacted with only one other species, B. thetaiotaomicron. Anti-B. fragilis serum reacted with a variety of species tested including Capnocytophaga (Bacteroides) ochraceus and F. nucleatum. Anti-B. melaninogenicus subspecies melaninogenicus reacted with other species of black-pigmenting Bacteroides and also one strain of B. fragilis. / The reaction of sera from ANUG patients and age and sex matched healthy individuals with microbial isolates from the ANUG patients revealed no differences in the levels of IgG, IgA and IgM or the IgG antibody activity. The results also suggest that antigenic determinants are shared by the F. nucleatum isolates as well as the Bacteroides gingivalis isolates. / Dark-field microscopic analysis of ANUG plaque samples revealed an abundance of rods, cocci and spirochetes. The predominate spirochetes were the type with the "2-4-2" axial filament arrangement which are presumably Treponema denticola. A microscopic agglutination test demonstrated that cross-reactivity was observed within each of the species of spirochetes tested and that there was no sharing of surface antigens among T. pectinovorum, T. denticola and T. vincentii. SDS-PAGE analysis suggested that the three species demonstrate 25 to 35 proteins which share migrational similarities. When sera produced against T. denticola strains were reacted using enzyme-linked immunoelectro-transfer blot analysis with nitrocellulose sheets containing the antigens of 3 species, 27 to 30 antigens were identifiable in homologous reactions. In heterologous reactions approximately 14 to 19 cross-reactive antigens were observed. SDS-PAGE analysis of the outer sheath preparation for T. denticola strain W revealed a single major protein. / Studies were undertaken to determine if microorganisms isolated from ANUG lesions have specific binding receptors for corticosteroids. The results suggested that B. gingivalis has specific receptors for dexamethasone and cortisol whereas F. nucleatum and B. intermedius do not. Biopsies of the diseased tissues from two of the patients were obtained and the histopathologic studies suggest that a PMN infiltration is seen early after onset whereas the gingival tissue is infiltrated with lymphocytes as time after onset of the lesion increases.

Keywords: Serology; Antigens; Histopathology

ACUTE NECROTIZING ULCERATIVE GINGIVITIS: MICROBIAL AND IMMUNOLOGIC STUDIES

Annual/Final

(1/15/81-1/14/84)

William A. Falkler, Jr., Ph.D.

Supported by

U. S. Army Medical Research and Development Command

Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD-17-80-C-0181

University of Maryland Dental School

Baltimore, Maryland 21201

TABLE OF CONTENTS

TABLES

FIGURES

ACUTE NECROTIZING ULCERATIVE GINGIVITIS

A Brief Literature Review

In 1896 Vincent described an ulcerative infection of the gingival tissue which he believed to be caused by fusiform bacilli and spirochetal organisms (1). Orban (2) utilized the term acute necrotizing ulcerative gingivitis (ANUG) to describe this infection and his description follows. The patient usually presents with painful hemorrhagic gingivae, inability to partake of food or to brush his teeth, and often with general malaise. He may or may not be aware of increased salivation, a noisome odor and a metallic taste. There is ulceration and necrosis of the interdental papillae with possible extension to the marginal gingivae. Craters may be present with the loss of interdental papillae. The crateriform lesions are commonly covered with pseudomembranes and surrounded by erythematous borders. Fever and lymphadenopathy are common.

The etiology of the disease has not followed Koch's postulates in that exudate from an infected lesion has not produced ANUG lesions in humans and animals. Although fusospirochetal abscesses have been produced in experimental animals, the lesions are not similar to those of ANUG (2-5). Clinical lesions have been produced in animals only after first traumatizing the involved tissues (6-8). Other investigators have mentioned the importance of local factors such as erupting teeth, poor oral hygiene, poor margins on restorations, calculus build-up, poor contacts, occlusal factors and systemic factors such as physical exhaustion, emotional tension, nutritional deficiencies and metabolic disturbances (2,3,5,9-13).

Of these, emotional factors often appear before the onset of the disease regardless of the other factors (11,12,14,15). Although there have been epidemics of ANUG, the disease has been proven to be noncommunicable. The epidemics were due to the fact that the individuals affected were under the same adverse conditions, such as mental stress or altered living patterns (2,4,5,16-19). This disease has been well documented in military personnel (14,20,21).

Treatments of the disease have utilized chromic acid, nitrates, TCA, H_2O_2, sodium permanganate, mercurials, arsenicals and triamicinolene acetonide in an adhesive vehicle (4,9,18,22,23). Antibiotics have been and are still used as adjuncts to complete subgingival curettage, debridement and local corrective procedures (19-33). Also ultrasonic instruments have been used in routine periodontal procedures (34-37).

Early studies on the histopathology of ANUG lesions reported that the pathological process appears to begin on the surface of the epithelium. First, the keratin is destroyed, then the degeneration progressed through the various layers of the epithelium (including the basal layer and continues on into the immediate fibrous tissues). The adjacent tissues become edematous and infiltrated with inflammatory cells. On the surface of the lesion were found spirochetes, fusiform bacilli, cocci, and filamenting types (38-43). Spirochetes of large and intermediate size were shown capable of invading non-necrotic tissue of the ANUG lesion and the majority of these spirochetes appeared different from pure strains of cultivated Borrelia vincentii and Treponema microdentium (42). Electron microscopic observation of ANUG lesions allowed the observation of a bacterial zone containing numerous microorganisms, including various morphological types of spirochetes, a zone rich in neutrophiles, a zone of necrosis, a zone where larger spirochetes were observed within the tissues of the host in large numbers and to the exclusion of other organisms (42).

The participation of spirochetes in the etiology of fusospirochetal diseases still remains undetermined. Rosebury et al. (44-46) in a series of experiments using mixtures of 29 bacterial cultures and five spirochetal cultures were unable to elicit infections in guinea pigs different than that observed when using fusospirochetal exudate (46). Sixteen bacterial strains recombined with Treponema microdentium did not produce fusospirochetal abscesses in guinea pigs (47). Typical "fusospirochetal" abscesses have been observed in guinea pigs with a mixture of two strains of Bacteroides, a motile gram-negative rod and diphtheroid (48). In this system neither spirochetes nor fusiform bacteria were essential. Borrelia vincenti, Borrelia buccale and small oral treponemes

produced localized abscesses in rabbits (49). Spirochetal abscesses were also observed in the hamster cheek pouch after injection of Borrelia buccalis and small oral treponemes (50). Intracutaneous lesions which resulted in abscess formation were routinely produced in rabbits with both Fusobacterium nucleatum and Fusobacterium polymorphum alone or in combination with oral spirochetes. The synergistic combinations of fusobacteria and spirochetes in intracutaneous lesions in animals showed definite evidence of invasion of both types of organisms into the surrounding tissues (51). Sections of interdental papilla of Vincent's infection stained by the Warthin-Faulkner method revealed spirochetes and fusiform bacilli penetrating the tissue (52).

Cell-mediated immunity and humoral antibody studies were investigated in patients with acute ulcerative gingivitis using antigens from Actinomyces viscosus, Fusobacterium fusiform, Veillonella alcalescens and Bacteriodes melaninogenicus. No difference in serum antibody levels reactive to the antigens were observed between patients and controls. The significantly greater cell-mediated immunity to F. fusiforme in ANUG suggested this organism might be involved in the change from the chronic to the acute form of the disease (53).

When whole saliva samples from ANUG patients were examined Harding et al. (54) found a decreased total immunoglobulin concentration but an increased secretory IgA concentration as compared to the levels in saliva of individuals with healthy gingiva. Within one to four days after the onset of clinical symptoms serum IgG levels were shown to be decreased whereas IgM levels were increased as compared to controls. However the decreased IgG levels returned to normal levels in about one month (55).

No significant differences were observed in serum antibody levels between ANUG patients and control subjects when the serum was reacted with strains of B. melaninogenicus, Fusobacterium nucleatum, Actinomyces viscosus, Veillonella parvula and an oral spirochete (56). Significantly higher IgG and IgM titers were observed to intermediate-sized spirochetes and higher IgG titers to Bacteroides melaninogenicus subsp. intermedius in the sera of ANUG patients when compared to sera of healthy

individuals and those with gingivitis (57).

Immunologic studies of ANUG were undertaken to detect humoral antibody reactive with oral spirochetes and to ascertain if spirochetes in vivo were coated with IgG, IgA, IgM and C3. Sera taken at the acute stage of ANUG revealed low antibody titers to oral spirochetes and ranged from 0 to 80. Also in ANUG, spirochetes in smears from the lesions were coated in vivo with IgG, IgA, IgM and C3. The authors suggested these findings are indicative of a localized antibody production to oral spirochetes. It was suggested that the interaction of these antibodies with spirochetes and subsequent complement activation may contribute to the pathogenesis of ANUG (58).

Jacob et al. (59) isolated anaerobic spirochetes from patients and prepared rabbit antisera. The antisera detected, with the use of an indirect fluorescent antibody technique, oral spirochetes in dental plaque from 10 patients suggesting that a common antigenic determinant was shared by the spirochetes detected. These investigators have also isolated from oral spirochetes a sodium deoxycholate-ethanol extractable antigen which appears to be shared by several oral isolates and to which human antibody is reactive (60).

Bacteroides melaninogenicus subsp. intermedius was shown to be a prominent microorganism in ANUG by Slots and Zambon (61). Other black-pigmented Bacteroides, including B. gingivalis and B. melaninogenicus subsp. melaninogenicus occurred only in small proportions. Loesche et al. (62) anaerobically cultured plaque samples from 22 ulcerated sites in eight patients with ANUG. They observed a constant flora comprised of a limited number of bacterial types and a variable flora composed of a heterogeneous mixture of bacterial types. The constant flora included Treponema and Selenomonas sp., B. intermedius and Fusobacterium sp. Treatment with metronidazole resulted in a prompt resolution of clinical symptoms with a significant reduction in the numbers of Treponema sp., B. intermedius and Fusobacterium sp. for several months following treatment.

SUMMARY

This is the final report on a research project aimed at obtaining new information as to the microbial etiology and immunopathology of acute necrotizing ulcerative gingivitis (ANUG). Thirty five patients have been studied as of the date of this report. Of these 54.1% were male and 45.8% were female, 18 were caucasion and 17 were black. The mean age of the patients was 23 years with a range of 14 to 50 years. The patients upon entering the clinic felt that they were healthy with the chief complaint being "sore gums". Clinical observations of the patients included cratering of papilla, fetid odor, pseudomembrane formation, blunting of papilla and lymphadenopathy. Thirty nine percent of the ANUG patients were febrile. Spontaneous gingival bleeding was observed in 66.6% of the patients. The most frequent sextant of the mouth affected by ANUG was the mandibular anterior region. Generalized ANUG was observed in 24.2% of the patients.

Forty percent of the patients were unemployed, those employed had jobs primarily of a service or technical support nature. Only 35% of the employed ANUG patients were satisfied with their present job and 46% felt that they were not working at a job for which they were best suited. Eighty three percent of the patients smoked. Over half of the patients (57%) reported sleeping restfully for 6-8 hours per night.

Subgingival plaque samples taken from the patients revealed the presence of large numbers of spirochetes and Gram negative rods. Cultural studies showed that the Gram negative rods comprised the highest percentage (78%) of the total organisms isolated. Of this group Bacteroides gingivalis and Fusobacterium nucleatum were the most frequently isolated with Bacteroides intermedius, other Fusobacterium sp. and Vibrio sp. also being identified. The Gram positive cocci comprised 15.5% of all of the isolates, Streptococcus and Staphylococcus sp., Pseudomonas micros and Peptostreptococcus sp. being isolated. Gram positive rods identified include Clostridium beijerinckie, Lactobacillus fermentum and Actinomyces israelii. Veillonella parvula was the only

identified Gram negative coccus isolated from these patients.

Studies were undertaken to determine possible colonization and pathogenic mechnaisms of B. gingivalis and F. nucleatum in the gingival crevice. Tests were performed which displayed the characteristic hemagglutination activity of the isolates. Experiments were performed to determine whether hemolysis would occur following attachment of strains of F. nucleatum and Bacteroides species including B. gingivalis. The F. nucleatum strains consistently displayed both hemagglutination and hemolytic activity. The B. gingivalis strains and other Bacteroides species displayed hemagglutination but no measurable hemolytic activity. Varying the concentration of the F. nucleatum whole cells in the standard hemolysis assay suggested a F. nucleatum-erythrocyte binding site interaction. The hemolytic moiety was observed in various cells, cell wall and lipopolysaccharide extracts. The lysis of erythrocytes by F. nucleatum may allow the release of iron-containing metabolites for other microorganisms and may provide a means of cytotoxicity to leukocytes, gingival fibroblasts and crevicular epithelial cells to which it attaches.

F. nucleatum, F. periodonticum (33693) and F. nucleatum isolates from individuals with chronic periodontitis, juvenile periodontitis, ANUG and healthy gingiva were compared serologically to each other and to other Fusobacterium species and F. nucleatum isolated from canines and a Macaca mulatta monkey. All F. nucleatum and F. periodonticum strains displayed biochemical reactions similar to those of the typed strains in API 20A biochemical testing. The human and monkey isolates displayed both type I and type II colonial morphologies on crystal violet erythromycin agar, whereas the canine isolates displayed only type I. Antigen preparations of the human isolates, typed strains, F. periodonticum and two of the monkey strains shared lines of identity in immunodiffusion testing. None of the other Fusobacterium species reacted with rabbit or Guinea pig antisera in immunodiffusion testing. As observed in immunodiffusion testing, all human isolates of F. nucleatum, the typed strains and F. periodonticum

shared antigens when reacted with rabbit anti-F. nucleatum sera in immunoelectrophoresis. An enzyme-linked immunosorbent assay in which rabbit anti-F. nucleatum and human sera were used revealed that the human and monkey isolates when used as antigens, allowed the detection of similar degrees of antibody activity; however, no activity was observed with the canine isolates. None of the canine isolates displayed the characteristic hemagglutination of sheep erythrocytes demonstrated by the human and monkey strains. These results suggest that F. nucleatum isolates from various human periodontal diseases, including ANUG, share antigenic determinants. These findings are important when considering the immunopathological mechanisms involved in human periodontal diseases and when comparing animal model systems with naturally occurring disease in humans.

Antisera were produced reactive with Bacteroides fragilis, B. gingivalis, or B. melaninogenicus subspecies melaninogenicus. These antisera were used in radial immunodiffusion testing to examine the serological reactivity of sonicated antigen preparations of 43 strains of various species of Bacteroidaceae, especially the B. fragilis group and black-pigmenting Bacteroides. The antisera were also adsorbed with homologous whole cells and used in radial immunodiffusion to examine antigens shared with non-homologous species. Anti-B. gingivalis serum exhibited strong reactions with the strains of homologous species and reacted with only one other species, B. thetaiotaomicron. Anti-B. fragilis serum reacted with a variety of species tested including Capnocytophaga (Bacteroides) ochraceus and Fusobacterium nucleatum. Anti-B. melaninogenicus subspecies melaninogenicus reacted with other species of black-pigmenting Bacteroides and also one strain of B. fragilis. This study suggests radial immunodiffusion can be useful in the identification of B. gingivalis. Antigens shared by B. fragilis and B. melaninogenicus subspecies of the Bacteroides must be considered when serological methods are used for their identification.

The reaction of sera from ANUG patients and age and sex matched healthy

individuals with microbial isolates from the ANUG patients revealed no differences in the levels of IgG, IgA and IgM or the IgG antibody activity. The results also suggest that antigenic determinants are shared by the Fusobacterium nucleatum isolates and that antigens are also shared by the Bacteroides gingivalis isolates. Serologic studies using these isolates and the sera are still being performed in our laboratory.

Dark-field microscopic analysis of ANUG plaque samples revealed an abundance of rods, cocci and spirochetes, representing 42.7%, 29.0% and 29.9% of the microorganisms, respectively. The predominate spirochetes appear to be the type with the "2-4-2" axial filament arrangement which are presumably Treponema denticola. The next most abundant spirochetes were equally divided among the large size spirochetes of the "12-24-12", "6-12-6" and "8-16-8" classes. Scanning electron microscopic analysis as well as videotapes using dark-field and phase contrast optics demonstrated the predominate flora to be spirochetes. A microscopic agglutination test demonstrated that cross-reactivity was observed within each of the three species of spirochetes tested and that there was no sharing of surface antigens among T. pectinovorum, T. denticola and T. vincentii. SDS-PAGE analysis suggested that the three species demonstrate 25 to 35 proteins which share migrational similarities. When sera produced against T. denticola strains W and MS were reacted using enzyme-linked immunoelectro-transfer blot analysis with nitrocellulose sheets containing the antigens of all 3 species, 27 to 30 antigens were identifiable in homologous reactions. In heterologous reactions approximately 14 to 19 cross-reactive antigens were observed. Also it was shown that proteins of several oral spirochete species including T. denticola, adsorb rabbit albumin. SDS-PAGE analysis of the outer sheath preparation for t. denticola strain W revealed a single major protein. Electron microscopy of the outer sheath preparation revealed large sac-like vessicles carrying a mosaic-like array.

Studies were undertaken to determine if microorganisms isolated from ANUG lesions have specific binding receptors for corticosteroids. The results suggested that B.

gingivalis has specific receptors for dexamethasone and cortisol whereas F. nucleatum and B. intermedius do not. Biopsies of the diseased tissues from two of the patients were obtained and the histopathologic studies suggest that a PMN infiltration is seen early after onset whereas the gingival tissue is infiltrated with lymphocytes as time after onset of the lesion increases.

ANUG PATIENTS

Clinical Evaluation and History

Thirty five patients with ANUG have been studied. The clinical evaluation of the patients is shown in Table 1.

The patients were asked to fill out an "ANUG History" questionaire. The answers were as follows:

1. What is your present job:

   Forty percent of the patients were unemployed at the time of coming to the clinic. The following jobs were listed:

   a. food service
   b. hair stylist
   c. barmaid
   d. electronics
   e. operations manager
   f. students
   g. file clerk
   h. service station manager
   i. bakery worker
   j. clerk
   k. dancer
   l. dental hygienist
   m. insurance broker
   n. family counselor

2. Are you satisfied with your present job?

   Only 35% were satisfied with their present job.

3. Living conditions:

   20 or 57.1% lived at home with their family
   7 or 20.0% lived alone
   8 or 22.8% lived in an apartment with a roommate

4. Nineteen or 54.2% of the patients were male; 16 or 45.8% were female

5. The mean age of the patients was 23 with a range of 14 to 50.

6. The average length of time that they lived at their current address was 54 months with a range of 1 week to 27 years.

7. The patients classified their own health status as follows:

   7 or 20.0% Excellent
   20 or 57.1% Good
   8 or 22.9% Fair

8. They came to the dental clinic because:

   23 or 65.7% Painful, bleeding gums
   4 or 11.4% Painful gums
   2 or 5.7% Painful tongue
   6 or 17.2% Other

9. Twenty six or 74.3% of the patients had sore gums when they entered the dental clinic.

10. When asked if the gums of the patients were painful prior to coming to the clinic:

    26 or 74.3% painful 5 days or more
    5 or 14.3% painful 4 days
    2 or 5.7% painful 3 days
    2 or 5.7% painful 2 days

11. When asked if their gums had ever been painful before:

    22 or 62.9% stated never
    8 or 22.8% stated once
    3 or 8.6% stated more than three times
    2 or 5.7% stated twice

12. When asked when was their last episode of painful gums: mean 26 months with a range of 1 month to 4 years ago.

13. When asked what they were doing at the time of the last episode:

    They stated they were either working, unemployed or that this was the first episode.

14. When asked if their gums bleed when they brushed their teeth:

    24 or 68.5% said yes; 11 or 31.5% said no.

15. When asked if their gums every bled by themselves - without brushing:

    22 or 62.8% said no; 13 or 37.2% said yes

16. When asked how long their gums had been bleeding prior to their coming to the clinic:

    2 or 5.7% no previous bleeding
    4 or 11.4% one day
    5 or 14.3% two days
    3 or 8.5% three days
    3 or 8.5% four days
    18 or 51.6% five days or more

17. When asked if this spontaneous bleeding ever happened before:

    25 or 71.4% stated never
    2 or 5.7% stated once
    8 or 22.9% stated more than 3 times

18. The last spontaneous bleeding episode of the patients was: mean 6 months with a range of one-12 months

19. When asked what they were doing during their last episode:

    Working, unemployed or in school

20. When asked if they smoke:

    29 or 82.8% did smoke; 6 or 17.2% did not

21. When asked how much they smoked:

    24 or 68.6% 0-1 pack/day
    6 or 17.1% 1-2 packs/day
    5 or 14.3% over 2 packs/day

22. When asked how much sleep they had per night the week prior to visiting the clinic:

    4 or 11.4% 0-4 hours
    12 or 34.3% 4-6 hours
    14 or 40.0% 6-8 hours
    5 or 14.3% over 8 hours

23. When asked how much sleep they had per night this last month:

    -- 0-4 hours
    5 or 14.3% 4-6 hours
    20 or 57.1% 6-8 hours
    10 or 28.6% over 8 hours

24. When asked if they sleep restfully when they do sleep:

    25 or 71.4% stated yes; 10 or 28.6% said no

25. When asked if they were working at a job for which they were best suited:

    19 or 54.3% stated yes; 16 or 45.7% stated no

26. When asked how many hours per day they were at their job:

    4 or 11.4% 0-2 hours
    5 or 14.3% 2-4 hours
    ---- 4-6 hours
    10 or 28.5% 6-8 hours
    13 or 37.1% 8-10 hours
    3 or 8.7% over 10 hours

27. When asked how many days per week they were at their job:

3 or 8.5% 0-2 days
1 or 2.9% 3 days
7 or 20.0% 4 days
20 or 57.1% 5 days
2 or 5.6% 6 days
1 or 2.9% 7 days

28. When asked how many meals they ate each day:

6 or 17.1% 1 meal
17 or 48.6% 2 meals
8 or 22.8% 3 meals
4 or 11.5% more than 3 meals

29. When asked how many meals they ate in a "fast food" establishment: Of the 50% which ate in fast food restaurants:

18 or 51.4% ate one meal
11 or 31.4% ate two meals
6 or 17.2% ate three meals

30. When asked if they felt they ate well:

24 or 68.6% stated yes; 11 or 31.4% stated no

31. When asked if they have time to brush their time:

33 or 94.3% answered yes; 2 or 5.7% no

32. When asked how often they brush their teeth each day:

4 or 11.4% less than once per day
12 or 34.4% once per day
13 or 37.1% twice per day
4 or 11.4% three times per day
2 or 5.7% never

33. When asked how often they floss each day:

3 or 8.6% less than once per day
6 or 17.1% once per day
3 or 8.6% twice per day
1 or 2.8% three times per day
22 or 62.9% never

34. When asked if the patients felt they had enough time to accomplish their responsibilities:

23 or 65.7% stated yes; 12 or 34.3% no

35. When asked if they enjoyed their current living situation:

22 or 62.8% stated yes; 13 or 37.2% no

36. When asked if they enjoyed their present job:

    20 or 57.1% stated yes; 15 or 42.9% no

37. When asked if the present condition of their mouth affected their work:

    20 or 57.1% stated yes; 15 or 42.9% no

38. Race

    18 caucasion; 17 black

Table 1
ANUG Patients

Patient #	Gingival Bleeding S	Gingival Bleeding P	Blunting of Papilla	Loss of Papilla 1/3,1/3-2/3, 2/3			Pain	Fetid Odor	Cratering of Papilla	Pseudo-membrane	Lymph-adenopathy	Onset	Temp.	Area Affected
1	+		+	+			+	+	+	+	+	2 weeks	99.2	Mand. Anterior
2		+	+			+	+	-[a]	+	+	-[a]	1 month	--[c]	Mand. Ant. Max. Post
3	+		+			+	+	+	+	+	+	--	--	Generalized
4		+	--[a]	+			+	+	+	±[b]	+	5 months	--	Max. Ant. Mand. Ant.
6	+		+				+	+	+	+	--	--	--	Mand. Ant.
7		+	--		+		+	+	±	±	--	3 days	--	Mand. Ant.
8	+		+			+	+	+	+	+	--	3 days	--	Mand. Ant.
9	+		--				+	+	+	+	--	1 week	--	Max. Ant. Max. Post.
10		+	--				+	+	+	+	+	1 week	99.6	Generalized
11	+		+		+		+	+	+	+	--	5 days	--	Generalized
12		+	±[b]	+			+	+	+	±	--	1 week	--	Generalized
13	+		--	+			+	+	+	+	+	1 week	--	Mand. Ant. Max. Ant.
14		+	--	+			+	+	±	±	--	4 days	--	Mand. Ant. Mand. Post.
15	+		+	+			+	+	+	+	--	4 days	--	Mand. Ant.
16	+		--		+		+	+	+	+	+	3 days	--	Mand. Ant.

Table 1 (con't.)

17	+		+		+		+	+	+	+	--	2 weeks	--	Mand. Ant.
18		+	--	+			+	+	+	+	--	1 week	--	Mand. Ant. Mand.Max.Post
		+												
19			+		+		+	+	+	+	+	2 days	102	Mand. Ant.
20	+		+		+		+	+	+	+	--	3 days	99.6	Generalized
21	+-	+	+			+	+	+	+	+	+	2 weeks	99.5	Mand. Ant. Max. Ant.
22		+	+	+			+	+	+	+	+-	4 days	--	Max. Ant.
23	+		+	+			+	+	+	-	+	1 day	100.1	Mand. Ant.
24	+		+		+		+	+	+	+	+	2 days	100.1	Max. Ant. Entire Mouth
25	+					+	+	+	+	+	+	1 day	100.0	Mand. Ant. Mand. Post
26	+		+			+	+	+	+	+	+	1 day	99	Mand. Ant. Mand. Post
27	+		+			+	+	+	+	+	+	1 day	100	Max. Ant.
29		+	+		+		+	+	+	+	+	2 days recurrent	--	Mand. Ant. Max. Post
30	+		+			+	+	+	+	+	+	1 day	99.2	Entire Mouth
31		+	+			+	+	+	+	+	-	6 months recurrent	99.0	Entire Mouth
32	+		+			+	+	+	+	-	+	1-2 days	--	Max. Ant.
33	+		$\pm^{b}$	+	+		+	+	+	+	+	1-2 days	--	Mand. Ant. Mand. Post.

Table 1 (con't.)

34	+	+	+	+	+	+	+	+	+	1 day	--	Mand. Ant. Post. Max. Ant.
35	+	+		+	+	+	+	+	+	1 day	99.1	Mand. Ant.

[a] no
[b] slight
[c] normal

Table 2
ANUG Patients
Summary of Clinical Findings

Clinical Parameter	% of total patients positive
Gingival Bleeding	
Spontaneous	66.6
Provoked	33.3
Blunting of Papilla	72.7
Loss of Papilla	
1/3	33.3
1/3 - 2/3	30.3
2/3	24.2
Pain	100.0
Fetid Odor	96.9
Cratering of Papilla	100.0
Pseudomembrane	84.8
Lymphadenopathy	60.6
Onset	
1-2 days ago	36.4
3-7 days ago	39.4
2 weeks ago	9.0
1-6 months ago	15.2
Elevated Temperature	39.4
Area of Mouth Affected	
Mandibular Anterior	63.7
Maxillary Anterior	27.3
Mandibular Posterior	18.2
Maxillary Posterior	12.1
Generalized	24.2

Of the individuals in this study 54.2% were male and 45.8% were female. The mean age of the ANUG patient was 23 years with a range of 14 to 50 years. Most (57.1%) of the patients reported that they were in "good" health with 20% reporting excellent health and 22.9% reporting as in fair health. Their chief complaints were "sore gums" (74.3%), painful, bleeding gums (65.7%), painful gums (11.4%), painful tongue (5.7%) and other oral problems (17.2%). The majority of the patients (74.3%) had pain in the gingival area for 5 days or more, 14% were painful 4 days, 5.7% were painful for 3 days, and 5.7% were painful for 2 days. The majority of the patients (62.9%) had never had pain before onset of initial symptoms, 22.8% had pain once prior to presenting, 5.7% had pain twice, and 8.6% had pain 3 or more times previously.

Forty percent were unemployed at the initial clinic visit. Of the patients employed, the jobs were primarily of a service or technical support nature (e. g. bar maid, file clerk, service station attendant, dancer, etc.). Only 35% of the employed ANUG patients were satisfied with their present job status. In addition, approximately one half (45.7%) of the patients felt that they were not working at a job for which they were best suited. Cigarette smoking appears to be a contributing factor in ANUG; 82.8% of patients smoked. Most patients (57.1%) reported sleep between 6-8 hours per night and 40% slept between 6-8 hours the night before onset of symptoms. Most (71.4%) stated that their sleep was restful.

Each (100%) ANUG patient presenting to the clinic reported pain. Additional clinical observations of the patients included cratering of papilla (100%), fetid odor (96.9%), pseudomembrane formation (84.8%), blunting of papillae (72.7%) and lymphadenopathy (60.6%). Thirty nine per cent of the ANUG patients were febrile with a mean temperature of 99.7. Spontaneous gingival bleeding, although reported by only 37.2% of patients was observed by the examiner in 66.6% of patients. However, bleeding with toothbrushing was a positive response for 68.5% of all ANUG patients suggesting the presence of an underlying periodontal disease. It was of interest that none of the

patients flossed regularly. The most frequent sextant of the mouth affected by ANUG was the mandibular anterior region (63.7%) followed by maxillary anterior (27.3%), mandibular posterior (18.2%) and maxillary posterior (12.19%). Generalized ANUG was found in 24.2% of all patients.

The patients in the present study were from a large metropolitan civilian area. Social profiles of this patient appear to be different from college students and military personnel. Many clinical observations such as fetid odor, cratering of papillae, pseudomembrane are similar to previously published reports, however, other signs such as lymphadenopathy and spontaneous gingival bleeding appear to be very prevalent in our population. Daily habitats such as smoking in our patients are consistent with previous reports. However, sleep patterns, which are rarely reported, suggest that our ANUG patients have adequate rest periods (6-8 hours/night).

CULTURING SUBGINGIVAL PLAQUE AND TISSUE SURFACE SCRAPINGS FOR MICROORGANISMS

Subgingival plaque and tissue surface scrapings have been taken from 30 ANUG patients. Seven of these samples were used in our initial studies in developing the cultural and identification procedures. Also attempts were made initially to isolate Fusobacterium nucleatum and Bacteroides strains from ANUG patients for use in the serologic studies to be presented. Plaque samples from the patients have revealed the isolation of approximately 8-12 different colony types from each patient. The procedure for culturing and identification of the samples follows:

Media

The following media were used: Crystal Violet-Erythromycin (CVE) agar; MM10 agar; and Trypticase Soy Agar (TSA) with hemin and menadione (TSAHK). CVE agar (pH 7.2 contains in g/l the following: trypticase (10.0), yeast extract (5.0), NaCl (5.0), Tryptophan (0.2), agar (15.0) and crystal violet (0.005), erythromycin (0.004), and defibrinated sheep blood (50 ml) which were added after autoclaving. MM10 contained/liter: H_2O (890 ml), 37.5 ml of 0.6% K_2HPO_4, 37.5 ml of a salt solution (NaCl), 1.2 g; $NH_4{}_2SO_4$, 1.2 g; KH_2PO_4, 0.6 g, Mg_2SO_4, 0.25 g and 100 ml H_2O), bacto-agar (15.0 g), trypticase (2.0 g), yeast extract (0.5 g), sucrose (30.0 g), KNO_3(0.25 g), 0.05% hemin, (2.0 ml) and cysteine (0.12 g), 8% Na_2CO_3 (5 ml), DL-dithio-threitol (0.1 mg) and sheep blood (20 ml) which were added after autoclaving. TSA contained 40 g/l trypticase soy agar and for the preparation of TSAHK 10 ml of 0.5 mg/ml hemin in 0.1 N NaOH, 1 ml of 0.1% menadione in 95% ETOH and 50 ml of defibrinated sheep blood were added.

Microbiological Culturing of ANUG Lesion

Samples of subgingival plaque were collected using a curette under nitrogen gas and immediately placed into 1 ml of reduced transort fluid (RTF) containing an Eh indicator (63). The vial was placed into a Coy anaerobic chamber, vortexed for 30 seconds and the

contents of the vial diluted in serial ten-fold dilutions with RTF. Each dilution (0.1 ml) was spread plated onto 3 reduced MM10 agar plates. Also 0.1 ml of the 10^{-1} dilution was spread plated onto CVE agar plates and TSAHK. The plates were allowed to incubate for 3 days, after which time they were inspected and the dilution showing 30-200 colonies/MM10 plate was used for quantitation of the viable count. The average of the colony counts for the 3 plates at that dilution was multiplied by the reciprocal of the dilution times 10. The number of colony types on each plate were observed and the average number of each colony type on the plates determined. The % of each colony type in relation to the total viable count was calculated. Each colony type was Gram stained and subcultured for isolation onto MM10 plates and incubated anaerobically for 2 days. Also each colony type was subcultured onto another MM10 plate and incubated aerobically for oxygen tolerance. Each isolate from MM10 was subcultured to BHI broth and allowed to grow 48 h and then placed into litmus milk and frozen. Anaerobes were identified by the API 20 anaerobe system. Facultative anaerobes and Gram positive cocci were identified by the API 20S system. Several streptococci were identified by their colonial morphology on MM10, for example, S. sanguis (transparent, shiny, immovable, rigid colonies), S. salivarius (large, transparent, sticky, mucoid colonies) and S. mutans (colorless, granular, indented, small colonies). Black pigmented colonies were taken from the TSAHK and subcultured for isolation on TSAHK and then identified via the use of the API 20A system. F. nucleatum colonies isolated from the CVE agar were also identified with the API 20A system. Sonicated suspensions of the isolates were also identified via reaction by immunodiffusion with rabbit antiserum to prototype strains.

RESULTS

The results of individual cultural studies on ten of the ANUG patients are presented in Tables 3-12. The results of the cultural studies of twenty of the patients have been combined and presented in Table 13. As can be observed in Table 13, to date the Gram negative rods comprised the highest percentage of the total organisms isolated. Of this

Table 3

ANUG I
White Male - Total counts on MM10 at 10^{-4}:
1. 90
2. 96

Avg. Total Viable Count (using 0.1 ml)
93×10^5

On MM10 (nonselective) medium:

Organism	Avg. Viable Count/ml	% Total Viable Count	ID GS	COL	API	SEL MED
Strep. intermedius	42×10^5	45.2		x		x
Strep. morbillorum	19×10^5	20.4		x		x
Bacteroides gingivalis	2×10^5	2.2		x	x	x
Fuso. nucleatum	2×10^5	2.2		x	x	x
Strep. sanguis	7×10^5	7.5		x	x	

Non-identified on MM10: classified according to Gram reaction and morphology:

Colonial Morph.	Gram Stain	O_2	Avg. Viable Cnt./ml	% Total Viable Cnt.	API 20A/20S
Med. brown with dk. ctr.	Gm variable cocci	(+)	1×10^5	1.1	20S - all neg.
Smooth, flat, shiny, greenish-gray	Gm (+) cocci chains	(+)	1×10^5	1.1	ND
Creamy-white, raised with Beta hemolysis	Gm (-) rods	(-)	7×10^5	7.9	20A - $S^-E^-C^+$
Tiny, beige, smooth, raised with reg. edge	Gm (-) cocci	(+)	12×10^5	12.9	N/A

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

Table 4

ANUG II

Black Female - Total counts on MM10 at 10^{-3}:
1. 152
2. 164

Avg. Total Viable Count (using 0.1 ml)
158×10^4

On MM10 (nonselective) medium:

Organism	Avg. Viable Count/ml	% Total Viable Count	ID GS	ID COL	ID API	SEL MED
Peptostrep. prevotii	92×10^4	58.2		x	x	
Veillonella parvula	8.5×10^4	5.4		x	x	
Peptostrep.	2×10^4	1.3		x	x	
Bacteroides gingivalis	1×10^4	0.7		x	x	x
Strep. sanguis	1×10^4	0.7		x	x	x
Fusobacterium spp.	23×10^4	14.61		x	x	x

Non-identified on MM10: classified according to Gram reaction and morphology:

Colonial Morph.	Gram Stain	O_2	Avg. Viable Cnt./ml	% Total Viable Cnt.	API 20A/20S
Mucoid, red & white, smooth round, shiny	Small Gm (-) cocci	(-)	1×10^4	0.7	20A - $S^+E^-C^-$ (Lac, Sac)
Flat, round, with dark brown center, white rim	Gm (-) coccobacilli	(-)	4×10^4	2.6	20A - $S^-E^-C^-$
Green, raised, irreg. edge	Gm (+) sequented rods	(-)	0.5×10^4	0.4	20A - $S^-E^+C^-$ (Ind, Esc)
Small, pink, irreg. edge, raised, rough surface	Branching, grainy Gm (+) rods	(-)	3.5×10^4	2.2	20A - $S^-E^-C^-$
Dark brown, irreg. edge, raised surface	Gm (+) diptheroid-like rods	(+)	10×10^4	6.3	N/A
Shiny, smooth, round, irreg. edge	Shant, plump Gm (-) coccobacilli	(-)	1×10^4	0.7	20A- $S^-E^-C^-$

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

Table 5

ANUG III

White Male - Total counts on MM10 at 10^{-3}:

1. 231
2. 274
3. 164

Avg. Total Viable Count (using 0.1 ml) 223×10^4

On MM10 (nonselective) medium:

Organism	Avg. Viable Count/ml	% Total Viable Count	ID GS	COL	API	SEL MED
Fusobacterium nucleatum	33×10^4	14.8		x	x	
Veillonella parvula	4.3×10^4	1.9		x	x	
Strep. morbillorum	0.3×10^4	0.1		x	x	
Strep. sanguis	0.7×10^4	0.3		x	x	
Veillonella parvula	1.3×10^4	0.6		x	x	

Non-identified on MM10: classified according to Gram reaction and morphology:

Colonial Morph.	Gram Stain	O_2	Avg. Viable Cnt./ml	% Total Viable Cnt.	API 20A/20S
Purple, raised center, pink rim, shiny, irreg. edge	Gm variable coccobacilli	+	4×10^4	1.8	20S - all neg
Dark red with pink rim, irreg. edge	Gm (+) rods short, plump	+	13.3×10^4	6.0	N/A
White, shiny, raised center	Short, plump Gm (-) rods	+	0.3×10^4	0.1	N/A
Small, dark, reddish-brown, convex	Long Gm (-) rods in short chains	-	16×10^4	7.2	N/A
Tiny, brown, smooth	Small Gm (-) rods	-	89×10^4	39.9	20A - $S^+E^-C^-$ (Lac, Sac, Mal, Rha)

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

Table 6

ANUG XIV
22 YOBM - Total counts on MM10 at 10^{-4}:
1. 135
2. 99
3. 100

Avg. Total Viable Count (using 0.1 ml) 111.3×10^5/ml

On MM10 (nonselective) medium:

Organism	Avg. Viable Count/ml	% Total Viable Count	ID GS	COL	API	SEL MED
Bacteroides gingivalis	6.0×10^5	5.4		x	x	x
Fusobacterium nucleatum	5.0×10^5	4.5		x	x	x
Clostridium beijerinckii	0.6×10^5	0.6		x	x	
Streptpcoccus sanguis	1.0×10^5	0.9		x	x	

Non-identified on MM10: classified according to Gram reaction and morphology:

Colonial Morph.	Gram Stain	O_2	Avg. Viable Cnt./ml	% Total Viable Cnt.	API 20A/20S
White, raised, shiny smooth with regular edge	Gm (-) cocci in and chains	(+)	3×10^5	2.7	API 20S - all neg.
Raised, shiny mucoid smooth with regular edges	Gm (+) cocci in clusters	(+)	11×10^5	9.9	API 20S - all neg.
Black-pigmented raised with irrreg. edge. Smooth; lighter brown periphery.	Gm (-) cocco bacillus	(+)	6×10^5	5.4	N/A
Light brown smooth center with rough dark brown periphery	Gm (-) cocci	(+)	2.3×10^5	2.1	API 20S - all neg.
Brown, rough, flat colony with irreg. edge. Spreader	Gm (-) rods	(+)	1×10^5	0.9	N/A
White, smooth, mucoid colony with reg. edges, center raised to peak	Gm (-) long fusiform rods	(+)	2×10^5	1.8	N/A

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

Table 7

ANUG XV
White Female - Age 42

Total counts on MM10 at 10^{-4}:
1. 60
2. 89
3. 99

Avg. Total Viable Count (using 0.1 ml)
82.6×10^5

On MM10 (nonselective) medium:

Organism	Avg. Viable Count/ml	% Total Viable Count	ID GS	COL	API	SEL MED
Ps. micros	2×10^5	2.42		x	x	x
F. nucleatum	12.5×10^5	1.51		x	x	x
S. sanguis	1×10^5	1.21			x	

Non-identified on MM10: classified according to Gram reaction and morphology:

Colonial Morph.	Gram Stain	O_2	Avg. Viable Cnt./ml	% Total Viable Cnt.	API 20A/20S
Raised, shiny, brown light brown periphery, reg. edge	Gm (+) rods w/terminal spores	(+)	12×10^5	14.5	NI
white, raised, shiny, peaked center, reg. edge	Gm (+) cocci	(+)	3×10^5	3.63	20S - all neg.
White, dull, raised, center peak, irreg. edge	Gm (+) cocci	(+)	3×10^5	3.63	20S - (+) Leu
dark brown, pinpoint, raised, reg. edge	Gm (-) short blunt rods	(-)	13×10^5	15.7	20A - $S^+E^-C^-$ (Ind, Glu, Sac, Gel)
small, creamy, irreg. edge, light brown center peak, raised	Gm variable segmented branching rod	(+)	3.5×10^5	4.2	N/A
small, white, raised, reg. edge, shiny	Gm (-) cocci	(-)	7.3×10^5	8.8	20A - $S^+E^-C^-$ (Glu, Lac, Sac, Mal, Gly, Cel, Raf, Sor, Trc)

Table 7 con't.

On TSAHK

Organism at 10^{-3}		
NI	I Black pigmenting colony	API 20A - $S^+E^-C^-$ (Glu, Lac, Sac, Mal)

On CVE at 10^{-3}

Organism	GS	COL	API	SEL MED
F. nucleatum	x	x	x	x
F. nucleatum	x	x	x	x
F. nucleatum	x	x	x	x

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

Table 8

ANUG
White Female - 12 years

Total counts on MM10 at 10^{-5}:
1. 75
2. 72
3. 78

Avg. Total Viable Count (using 0.1 ml)
75×10^6

On MM10 (nonselective) medium:

Organism	Avg. Viable Count/ml	% Total Viable Count	GS	ID COL	ID API	ID SEL MED
F. nucleatum	4×10^6	5.3		x	x	x
S. sanguis	1×10^6	1.3			x	
Ps. anaerobotus or micros	$.5 \times 10^6$	.67		x	x	x

Non-identified on MM10: classified according to Gram reaction and morphology:

Colonial Morph.	Gram Stain	O_2	Avg. Viable Cnt./ml	% Total Viable Cnt.	API 20A/20S
White, raised, smooth, shiny, reg. edge, large	Gm (-) thin fusiform rods	-	2×10^6	2 7	20A - $S^+E^-C^-$, Glu Man, Lac, Sac, Mal, Raf, Sor
Small, raised, tan, reg. edge	Gm (-) rods, med. length, thick, chains, curved	-	29×10^6	38.7	20A - $S^+E^-C^-$ - Glu, Man, Lac, Sac, Mal
Brown, raised, rough, irreg. edge, raised clear center	Gm (+) cocci	+	1×10^6	1.3	20S - all neg.
Brown, spreader, irreg. edge	Gm (-) rods, large thick, chains, w/ segmentation & single	-	1×10^6	1.3	20A - $S^+E^-C^-$ Glu, Lac, Sac, Mal, Sal, Ccl, Mne, Raf, Tre
Brown, clear edge, raised dark center, reg. edge	Gm (-) rods, large, thick, chains, w/ segmentation & single	-	1×10^6	1.3	20A - $S^+E^-C^-$, Glu, Man, Lac, Sac, Mal, Mne, Raf, Sor, Tre
Small, white, reg. edge, raised	V. small Gm (-) rods, comma shaped	-	3×10^6	4	20A - $S^+E^-C^-$ - Glu, Man, Lac, Sac, Mal

Table 8 con't.

	Avg. Viable Count	% of Total Black-Pigmenting Organisms	ID GS	ID COL	ID API	ID SEL MED
On TSAHK						
Bacteroides gingivalis	2×10^5		x	x	x	
On CVE						
F. nucleatum	7×10^5		x	x	x	

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

Table 9

ANUG IX

Total counts on MM10 at 10^{-4}:
1. 102
2. 61
3. 60

Avg. Total Viable Count (using 0.1 ml) 74.3×10^5

On MM10 (nonselective) medium:

Organism	Avg. Viable Count/ml	% Total Viable Count	GS	ID COL	ID API	ID SEL MED
Clostridium beijerinckii	24×10^5	32.3		x	x	
Bacteroides gingivalis	10×10^5	13.5		x	x	x
Fusobacterium mortiferum	2.3×10^5	3.1		x	x	
Lactobacillus fermentum	5.0×10^5	6.7		x	x	
Peptostrep. prevotii	1.3×10^5	1.7		x	x	
Vibrio sp.	1×10^5	1.3		x	x	x

Non-identified on MM10: classified according to Gram reaction and morphology:

Colonial Morph.	Gram Stain	O_2	Avg. Viable Cnt./ml	% Total Viable Cnt.	API 20A/20S
Opaque, raised center, flat periph. center smooth, irreg. wrinkled edge	Gm (-) med. length rods	(+)	4.3×10^5	5.8	N/A
Smooth flat colonies with slightly raised center. Center dark brown, periph. lt. brown edge regular	Gm (-) coccobacilli	(+)	3.3×10^5	4.4	N/A
Rough, raised, opaque Irreg. edge. Immovable	Thin Gm (-) rods Branched & sequented	(+)	2×10^5	2.7	N/A
Flat white rough red. edge	Small, thin Gm (-) rods	(-)	1×10^5	1.4	Died
Pinpt. dark brown rough with irreg. edge	Gm (-) cocci, somewhat kidney-shaped mostly pairs	(+)	0.3×10^5	0.4	N/A

On CVE (selective for detection of presumptive F. nucleatum strains)
Two strains of F. nucleatum isolated & ID via GS, phase, API & growth (colonial morph)

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

Table 10

ANUG XI
18 YO WM - Total counts on MM10 at 10^{-5}:
1. 77
2. 68
3. 38

Avg. Total Viable Count (using 0.1 ml)
61×10^6/ml

On MM10 (nonselective) medium:

Organism	Avg. Viable Count/ml	% Total Viable Count	ID GS	ID COL	ID API	ID SEL MED
Streptococcus sanguis	0.3×10^6	0.5			x	x
Actinomyces israelii	3.3×10^6	5.4		x		

Non-identified on MM10: classified according to Gram reaction and morphology:

Colonial Morph.	Gram Stain	O_2	Avg. Viable Cnt./ml	% Total Viable Cnt.	API 20A/20S
Shiny, tan, slightly raised with clear rim and reg. edge	Gm (-) short blunt rods	(-)	8×10^6	13.1	20A - $S^+E^-C^-$ - Ind, Gly, Lac, Sac, Mal, Xyl, Raf, Rha
Brown with slightly white peaked center. Rough, immovable with irregular edge	Gm (-) shant blunt coccobacillus	(+)	4×10^6	6.6	20A - All neg.
Rough star-shaped with white center and brown periphery - Immovable with irreg. edge	Gm (-) cocci	(+)	1×10^6	1.6	20S - All neg.
Small, translucent, shiny smooth & slightly raised	Gm (-) cocci	(+)	5.7×10^6	9.3	20S - All neg.
Violet colored smooth slightly raised rigid with regular edge	Gm (-) long thin rod	(-)	0.3×10^6	0.5	Died

Table 10 con't.

Rough, slightly raised and brown with scalloped edge	Gm (-) cocci	(+)	2×10^6	3.3	20S - All neg.
Raised, translucent center brown periphery. Center smooth; periph rough. Spreader	Large Gm (-) rod	(+)	0.3×10^6	0.5	N/A

On TSAHK (selective for black-pigmenting Bacteroides)

One strain of B. mel. ss intermedius was isolated & ID via GS, API 20A, colonial morph (pigment)

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

Table 11

19 YO BF - Total counts on MM10 at 10^{-4}: 1. 35 2. 34

Avg. Total Viable Count (using 0.1 ml) 34.5×10^5/ml

On MM10 (nonselective) medium:

Organism	Avg. Viable Count/ml	% Total Viable Count	ID GS	COL	API	SEL MED
Bacteroides gingivalis	12×10^5	34.8		x	x	x
Lactobacillus fermentum	7×10^5	20.3		x	x	
Fusobacterium varium	2.5×10^5	7.2		x	x	
Fusobacterium mortiferum	2×10^5	5.8		x	x	

Non-identified on MM10: classified according to Gram reaction and morphology:

Colonial Morph.	Gram Stain	O_2	Avg. Viable Cnt./ml	% Total Viable Cnt.	API 20A/20S
Beige mucoid with reg. edge	Thick Gm (-) rods med. length, blunt ends	(-)	8.5×10^5	24.6	20A - $S^+E^-C^-$
Dull fried egg appearance colony with dark brown center and light brown periphery	Branching, long thin segmented Gm (-) rods	(+)	4.5×10^5	13.1	N/A

On TSAHK (selective for black-pigmenting Bacteroides)

Two strains of Bacteroides gingivalis isolated and ID by GS, API 20A, colonial morph.

On CVE (selective for detection of presumptive F. nucleatum strains)

No F. nucleatum detected

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

Table 12

ANUG XIX
28 YO WM - Total counts on MM10 at 10^{-3}: Avg. Total Viable Count (using 0.1 ml)
JP superimposed 1. 43 39×10^4/ml
ANUG 2. 32
3. 42

On MM10 (nonselective) medium:

Organism	Avg. Viable Count/ml	% Total Viable Count	ID GS	COL	API	SEL MED
B. intermedius	15.7×10^4	40.3		x	x	x
B. gingivalis	14.7×10^4	37.7		x	x	x
F. necrophorum	2.0×10^4	5.1		x	x	
S. constellatus	0.7×10^4	1.8		x	x	

Non-identified on MM10: classified according to Gram reaction and morphology:

Colonial Morph.	Gram Stain	O_2	Avg. Variable Cnt./ml	% Total Variable Cnt.	API 20A/20S
Small, creamy, raised smooth with regular edges	Gm (+) branched rods long & thin & exhibiting segmentation	(-)	3.7×10^4	9.5	20A - $S^-E^-C^-$
Large, dark brown, dull, flat with regular edge	Gm (+) cocci in chains, clusters	(+)	0.7×10^4	1.8	20S - alpha hemolytic
Small, light brown center dark brown edge, flat, dull with irreg. edge	Gm (+) cocci in chains, clusters and pairs	(+)	0.3×10^4	0.8	20S - alpha hemolytic ARL - (+) Rest - (-)

On TSAHK (selective for black-pigmenting Bacteroides)

Organism	Avg. Viable Count/ml	% Total Viable Count of Black-Pigmenting Org's
B. intermedius	21.4×10^4	95.5
B. gingivalis	1.0×10^4	4.5

On CVE (selective for detection pf presumptive F. nucleatum strains)

No F. nucleatum detected

GS - Gram stain COL - colonial morphology API-20A or 20S SEL MED - selective media

Table 13

Levels of Suspected Odontopathic Organisms in Predominant Cultivable Flora of Plaque Taken from ANUG Sites of 20 Patients

		% of Total Quantitated Organisms			
I.	Gram-positive rods	2.80			
A.	Anaerobic	2.10	B.	Facultative Anaerobes	0.70
1.	Identified	1.70	1.	Identified 0.00	
	Clostridium beijerinckie	0.60			
	Lactobacillus fermentum	0.30			
	Actinomyces israelii	0.80			
2.	Unidentified	0.40	2.	Unidentified 0.70	
	$S^- E^- C^-$	0.03		(all non-hemolytic and ARL^-)	
	$S^+ E^- C^-$	0.26			
	$S^- E^+ C^-$	0.01			
	Other	0.10			
II.	Gram-positive cocci	15.50			
A.	Anaerobic	4.80	B.	Facultative Anaerobes	10.70
1.	Identified	2.60	1.	Identified 1.20	
	Pseudomonas micros	0.20		Streptococcus sanguis	0.40
	Peptostreptococcus sp.	0.30		Strep. sp. 0.40	
	Streptococcus sp.	2.10		Staphylococcus sp. 0.40	
2.	Unidentified	2.20	2.	Unidentified 9.50	
	$S^+ E^- C^-$	2.10		alphahemolytic, ARL^+ 7.10	
	Other	0.10		alphahemolytic, ARL^- 0.10	
				betahemoytic, ARL^- 0.60	
				nonhemolytic, ARL^+ 0.50	
				nonhemolytic, ARL^- 1.20	
III.	Gram-negative rods	78.20			
A.	Anaerobic	36.20	B.	Facultative Anaerobes	42.0
1.	Identified	12.50	1.	Identified 0.00	
	Bacteroides gingivalis	7.80			
	Bacteroides intermedius	0.10			
	Fusobacterium nucleatum	3.40			
	Fusobacterium sp.	1.10			
	Vibrio sp.	0.10			
2.	Unidentified	23.70	2.	Unidentified 42.0	
	$S^- E^- C^-$	1.40		(all nonhemolytic and ARL^-)	
	$S^+ E^- C^-$	11.30			
	$S^- E^- C^+$	0.20			
	Other	10.80			

			% of Total Quantitated Organisms		
IV.	Gram-negative cocci		3.50		
A.	Anaerobic	1.10	B.	Facultative Anaerobes	2.40
1.	Identified	0.80	1.	Identified	0.00
	V. parvula	0.80			
2.	Unidentified	0.30	2.	Unidentified	2.40
	$S^+ E^- C^-$	0.20		(nonhemolytic ARL^-)	
	Other	0.10			

S - saccharolytic
E - esculin
C - catalase

group B. gingivalis and F. nucleatum were the most frequently isolated with B. intermedius, other Fusobacterium sp. and Vibrio sp. also being identified. The Gram positive cocci comprised 15.5% of all of the isolates, Streptococcus and Staphylococcus sp., Peptostreptcoccus micros, and Peptostreptococcus sp. being isolated. A smaller number of Gram positive rods than expected was observed. These included isolates of C. beijerinckie, L. fermentum and A. israelii. V. parvula was the only identified Gram negative coccus isolated from these patients.

Discussion

There have been only a few attempts at cultivation of the microbial flora associated with the ANUG lesion. Prevoius studies by Rosebury et al. (44-46) and MacDonald (48) attempting to elicit infections in guinea pigs, "fusospirochetal" in nature suggested B. melaninogenicus as an essential pathogen. It is likely that the proteolytic black-pigmented bacteroides strains which MacDonald (48) studied would now be classified as B. gingivalis. Loesch et al. (62) reported in a study of eight patients that B. melaninogenicus ssp. intermedius and Fusobacterium sp., averaged 24 and 3%, respectively, of the viable count, B. gingivalis rarely being observed. Using an indirect fluorescent staining technique of plaque samples, Slots and Zambon (61) have also suggested B. intermedius as more numerous in the ANUG lesion that B. gingivalis. Our studies demonstrate B. gingivalis and Fusobacterium nucleatum as the major Gram negative isolates frm the patients sampled in this study. It is very possible that the presence or absence or degree of periodontal disease in the individual's mouth may have a direct influence on the type of flora present. If periodontal pockets are present there may be a shift from B. intermedius which could be associated with the clean oral cavity-college associated ANUG to B. gingivalis.

Hemagglutination Activity of ANUG Isolates

Oral strains of Fusobacterium nucleatum and Bacteroides gingivalis have been shown to attach to and cause hemagglutination (HA) of human and sheep red blood cells (64). This activity may be involved in the colonization and pathogenic mechanisms of these organisms in the gingival crevice (65). Studies were undertaken to see if Fusobacterium nucleatum and Bacteroides gingivalis and intermedius isolates from ANUG patients demonstrated HA activity.

Materials and Methods

Cultures and cultural conditions. The strains of Fusobacterium and Bacteroides used can be seen in Tables 14 & 15. The fusobacteria were grown in a modified tryptone medium (64) and the Bacteroides strains in Brain Heart Infusion broth supplemented with 5 g/ml hemin and 2 g/ml menadione using the BBL anaerobe jar-Gas Pak system. After 24 to 72 h of growth, the organisms were harvested by centrifugation at 10,000 x g for 10 min and washed three times with 0.15 M NaCl. The organisms were routinely resuspended in 0.01 M phosphate buffer containing 0.15 M NaCl and 0.2% sodium azide (PBS) at a concentration of approximately 0.28 g/ml or in a 10% suspension (packed volume, after centrifugation at 800 x g for 15 min, diluted 1:10 in PBS).

HA test. A modification of the microtiter test described by Crawford et al. (66) was used for HA testing. Twenty five microliters of the whole-cell suspensions were serially diluted twofold with microdiluters in a microtiter tray. To this was added 25 l of PBS followed by 25 l of a 3 x PBS-washed 1.25% sheep red blood cell suspension. The microtiter tray was shaken on a micromixer for 1 min and incubated at 37° C for 30 min followed by incubation at room temperature 2 h before reading. HA was recorded as 1 to 4+, 4+ being a smooth blanket of RBC covering the bottom of the well. PBS instead of the HA preparation was used as a control for normal buttoning of the RBC.

Results

As can be seen in Tables 14 & 15 the F. nucleatum strains displayed a wide range of HA activity whereas the Bacteroides strains displayed little if any HA activity.

Table 14

Hemagglutination Assay of F. nucleatum Isolates

F. nucleatum[a] strain	Hemagglutination Titer
VIII A_{14}	1:2
XII_3	1:8
WAF	1:1024
XA_{12}	1:4
VIII A_{13}	1:1024
CD_3	1:16
Lai	1:128
VI A_2	1:8
$XVII_8$	1:2
$XIII_2$	1:64
VI A_1	1:64
XVI_2	1:2
CD_2	1:4
Smoot	1:8
4355	1:1024
10197	1:64
10953	1:4
DS_1	1:1024
MR_3	1:8

[a] 5% whole cell suspensions
[b] dilution displaying at least 2+ HA

Table 15

Hemagglutination Assay of *Bacteroides* Isolates

Bacteroides[a] strains	Hemagglutination Titer
XII_8 *B. gingivalis*	1:2
CS44 *B. gingivalis*	-
CS43 *B. gingivalis*	-
CS41 *B. gingivalis*	1:2
XV_6 *B. gingivalis*	-
XIX_9 *B. intermedius*	1:2
25261 *B. intermedius*	-
382 *B. fragilis*	-
25285 *B. fragilis*	-
MH 678 *B. asaccharolyticus*	-
687 *B. asaccharolyticus*	-
B. ovatus	-

[a] 5% whole cell suspensions
[b] dilution displaying at least 2+ HA

Hemolytic Activity of F. nucleatum and B. gingivalis

The current studies were undertaken to determine if direct cell damage would occur following attachment of F. nucleatum and Bacteroides whole cells to human erythrocytes. This may participate in the development of the lesion observed in ANUG.

MATERIALS AND METHODS

Cultures and cultural conditions

Three strains of F. nucleatum were used in these studies: VPI 4355 and 10197 and ATCC 10953, originally designated F. polymorphum (67). Strains of B. thetaiotamicron (F-266), B. distasonis (F-24), B. fragilis (U-51) and B. ovatus (F-338) were supplied by Dr. Sharon Hansen, VA Hospital, Baltimore, Md. B. asaccharolyticus (MH-698) was supplied by Michael Hargadon, Baltimore Cancer Research Center, Baltimore, Md. Oral B. gingivalis strains (CS-41-44 and 47) were supplied by Dr. Carol Spiegel, VA Hospital, Wood, Wisconsin. All strains were tested for purity upon acquisition by culturing anaerobically on blood agar plates and examination by phase contrast microscopy. F. nucleatum strains were grown in a modified tryptone medium (64) or in Brain Heart Infusion (BHI) broth (Difco) supplemented with hemin (1 g/ml) and menadione (5 g/ml) (BHIKM). All Bacteroides strains were grown in the BHIKM medium. After incubation at 37° C for 48-72 h, using the BBL anaerobe jar-Gas Pak System (Baltimore Biological Laboratories, BBL , Cockeysville, Md.), the organisms were centrifuged at 10,000 x g for 15 min and the sedimented organisms washed 3x in 0.01 M phosphate buffer containing 0.15 M NaCl (PBS), pH 7.2, supplemented with 0.001 M $CaCl_2$ (PBS/Ca^{++}). The washed, sedimented organisms were resuspended in PBS to 10 percent suspensions (WCS) (68) and kept frozen until used in hemagglutination (HA) and hemolysis assays.

Standard hemolysis assay and preparation of the standard hemolysis curve

Hemoglobin standards were prepared using type A+ human erythrocytes (HEC) obtained from the Blood Bank, University of Maryland Hospital, Baltimore, Md. The A+

HEC were washed three times in PBS/Ca^{++} and the packed volume resulting from centrifugation at 600 x g for 5 min diluted 1:8 with PBS/Ca^{++} (1.25 per cent HEC suspension). Hemoglobin and hemolysis color standards were prepared according to the methodology in "Standard Diagnostic Complement Fixation Method and Adaptation to Micro Test" (Public Health Monograph #74). The optical density (OD) of the hemolysis test solutions was measured at 541 nm using a Gilford 2400 spectrophotometer and the zero percent hemolysis standard as a blank. All tests were performed in duplicate. Each time a fresh unit of human blood was obtained from the blood bank a new set of color standards was prepared and a new standard curve established.

Experimental conditions and hemolysis testing

Two fold serial dilutions of F. nucleatum 4355, 10197, and 10953 WCS in PBS/Ca^{++} (0.4 ml) were placed in conical centrifuge tubes. Each suspension was mixed with an equal volume of a 3x washed 2.5 percent suspension (PBS/Ca^{++}) of HEC (type A+ unless otherwise indicated). After gentle mixing, the tubes were incubated at 37° C for 1 h with mixing every 15 min followed by incubation at 4° C for 18 h. Preliminary experiments indicated this to be the optimal temperature and time for incubation. After gentle mixing and centrifugation at 600 x g for 5 min, 0.4 ml of the resulting supernatant fluid was pipetted into a small test tube and 1.6 ml of PBS/Ca^{++} added. The OD of the test solution was recorded and the per cent hemolysis determined from the standard hemolysis curve. This basic methodology was designated the standard hemolysis assay and was also used with WCS of the Bacteroides strains. The results were recorded as the average of duplicate samples unless more than a 5 per cent difference in hemolysis was observed between samples, in which case the experiment was repeated.

A variation of this procedure was used where F. nucleatum or Bacteroides strains were placed into tubes with HEC as above. However after incubation at 37° C for 15 min the HEC were sedimented by centrifugation at 600 x g for 5 min and after removal of the supernatant fluid, which would contain non-attached F. nucleatum or Bacteroides cells, resuspended in PBS/Ca^{++} to the original volume and incubated for 15 min at 37° C

followed by 4° C for 18 h. The suspensions were then treated as previously described for the hemolysis assay.

Experiments were performed to determine if the type of HEC would have an effect on the percent hemolysis by F. nucleatum strains. HEC types A+, 0+, 0-, and AB- were obtained from the Blood Bank, University of Maryland Hospital, Baltimore, Md and used to establish standard hemolysis curves. F. nucleatum 4355, 10197 and 10953 WCS were tested in the standard hemolysis assay using each of the HEC types and reading the percent hemolysis from the corresponding standard curve. All tests were run in duplicate.

WCS (2.0 ml) of F. nucleatum 4355, 10197 and 10953 were incubated in a water bath at various temperatures (56, 70, 86 and 100° C) for 1 h. After 30 min and at the end of 1 h, samples were removed from the water bath and tested in the HA and standard hemolysis assays. Samples of each 1 h treatment were also centrifuged at 10,000 g for 10 min and the resulting supernatant fluids tested for HA and hemolytic activity.

The WCS were also stored at 4° C for 10 weeks and once each week the hemoytic activity of the suspensions determined. All tests were performed in duplicate.

Sugar inhibition and F. nucleatum hemolysis

WCS (0.4 ml) of F. nucleatum 10197 and 10953 were centrifuged at 600 x g for 10 min. The resulting supernatant fluids were discarded, and the cells resuspended to 0.4 ml in PBS/Ca^{++} as a control or 100 and 200 mM solutions in PBS/Ca^{++} of D-glucose, D-galactose, D-lactose or D-raffinose. Duplicate samples were tested in the standard hemolysis assay.

Preparation of cell wall LPS extracts

F. nucleatum 4355, 10197 and 10953 WCS were heated at 70° C for 2 h and centrifuged at 10,000 x g for 15 min. The resulting supernatant fluids were collected and exhaustively dialyzed against distilled H_2O. The dialysates were lyophilized and approximately 7.0 mg of dry weight of each dissolved in 1.0 ml of PBS/Ca^{++} and tested

for HA and hemolytic activity. The cells resulting from the centrifugation step were resuspended in PBS/Ca^{++} and also tested in the HA and standard hemolysis assays.

LPS was prepared by a modification of the hot-phenol extraction of Weir (69). Briefly, F. nucleatum 4355, 10197 and 10953 6 per cent whole cell suspensions in distilled H_2O were preheated to 65° C in a water bath with continuous stirring. An equal volume of 90 percent preheated phenol was added and the mixture stirred for an additional five min. After cooling in an ice bath and centrifugation at 2,000 g for 1 h, the aqueous phase was separated and dialyzed against running tap water until no phenol odor could be detected. All aqueous phase preparations were lyophilized, reconstituted to approximately 7 mg dry weight per ml in PBS/Ca^{++} and tested for HA and hemolytic activity.

F. nucleatum 10953 WS were sonicated as previously described (68) and the sonicated suspensions centrifuged at 2,000 x g for 10 min. The crude cell walls were resuspended in distilled H_2O and treated by the method of Garcia et al. (70). The cell wall preparations were lyophilized and 7.5 mg dry weight of each sample resuspended in 1.0 ml of PBS/Ca^{++} and tested for HA and hemolytic activity.

An attempt was made to determine if the backbone of lipid A from F. nucleatum 4355 and 10197 would hemolyze type A+ HEC. The lipid A backbone was prepared by acid hydrolysis according to the method described by Hase, Hofstad and Rietschel (71). The lipid A backbone (7.0 mg dry weight/ml) was tested for HA and hemolytic activity and for HA activity in the presence of 100 mM D-galactose (Mongiello and Falkler, 1979).

RESULTS

F. nucleatum whole cell suspensions of the three strains were tested in the HA and standard hemolysis assays. The results, shown in Table 16, indicated that both the HA titer and per cent hemolysis increased as the concentration of whole cell suspensions

increased. Also a similar degree of lysis was observed with the HEC incubated only with F. nucleatum cells which were attached to their surface. The HA and hemolytic activity of F. nucleatum 10197 and 10953 WCS were decreased in the presence of D-galactose, D-lactose and D-raffinose but not D-glucose (Table 17).

Table 16. F. nucleatum HA and hemolytic activity

per cent whole cell suspension	HA Titer 10197	4355	10953	per cent hemolysis 10197		4355		10953	
10.0	256[a]	256	256	90[b]	86[c]	93	89	83	77
5.0	64	64	64	87		90		78	
2.5	32	32	32	42		45		35	
1.25	16	16	16	25		28		21	
0.625	16	8	8	18		16		8	
0.313	8	4	4	11		9		3	

[a] reciprocal of the highest dilution displaying a value of at least 1+ HA

[b] as determined by standard hemolysis assay

[c] hemolysis of cells incubated after removal of non-attached F. nucleatum

Table 17. Sugar inhibition of HA and hemolysis by F. nucleatum 10197 and 10953

		Hemagglutination		Hemolysis	
Solution	mM	10197	10953	10197	10953
D-glucose	200	128[a]	128	81[b]	87
		100	128	128	89
84					
D-galactose	200	+[c]	+	62	42
	100	+	+	77	53
D-lactose	200	+	+	18	20
	100	+	+	35	38
D-raffinose	200	+	+	16	18
	100	+	+	43	43
PBS/Ca^{++} control		128	128	89	85

[a] reciprocal of the highest dilution displaying at least 1+ HA

[b] as determined by the standard hemolysis assay

[c] complete inhibition of HA

Standard hemolysis curves for HEC types A-, 0+, 0- and AB- were established. F. nucleatum 4355, 10197, and 10953 WCS were tested in the standard hemolysis assay with the four blood types and the degree of lysis obtained from the corresponding standard curve. The results indicated that F. nucleatum suspensions showed hemolysis of all of the HEC tested irregardless of blood type.

The three F. nucleatum strains were heated at various temperatures in a water bath prior to use in the HA and standard hemolysis assays. Although the HA activity remained the same after treatment at 56 and 70° C, a slight increase in the hemolytic activity was observed (approximately 10 per cent). After heating at 86° C for 30 min, the F. nucleatum cells displayed at least two serial two-fold reductions in HA activity which increased to seven serial two-fold reductions following 100° C treatment for 30 min. The hemolytic activity also decreased with increasing heat treatment above 70° C and a greater than 50 per cent drop was noted after 100° C treatment for 30 min. During storage of the F. nucleatum strains at 4° C for 10 weeks the hemolytic activity varied by only plus or minus 5 per cent.

WCS of F. nucleatum and Bacteroides grown in BHIKM were tested for HA and hemolytic activity. The results (Table 17), indicated that all Bacteroides strains showed some degree of HA activity while none of the strains showed measurable hemolytic activity. F. nucleatum strains grown in BHIKM displayed both HA and hemolytic activity.

HA and hemolytic activity of F. nucleatum extract preparations

As can be seen in Table 19, the water phase LPS preparations of all three F. nucleatum strains demonstrated HA and hemolytic activity. The 70° C supernatant extracts also demonstrated HA activity with all strains however hemolysis was not observed with the preparation from strain 10197. The cell wall preparations demonstrated both HA and hemolysis activity.

Table 18. HA and hemolytic activity of Bacteroides and F. nucleatum strains grown in BHIKM.

Microorganism[a]	Designation	HA titer	per cent hemolysis
B. thetaiotamicron	F-266	2[b]	-[c]
B. asaccharolyticus	MH-698	0[d]	-
B. distasonis	F-244	8	-
B. fragilis	U-51	0	-
B. ovatus	F-338	16	-
B. gingivalis	CS-41	8	-
B. gingivalis	CS-42	8	-
B. gingivalis	CS-43	128	-
B. gingivalis	CS-44	8	-
B. gingivalis	CS-47	16	-
F. nucleatum	4355	512	93
F. nucleatum	10197	512	89
F. nucleatum	10953	256	76

[a] WCS

[b] reciprocal of the highest dilution displaying at least a 1+ HA

[c] no measurable hemolysis as determined by standard hemolysis assay

[d] undiluted

Table 19. HA and hemolytic activity of F. nucleatum 4355, 10197 and 10953 extract preparations

F. nucleatum strain #	Extract	dry weight mg/ml in PBS/Ca^{++}	HA[a]	per cent hemolysis[b]
4355	LPS-water phase	6.8	16	50
"	70° C supernatant	7.0	32	29
10197	LPS-water phase	7.0	32	98
"	70° C supernatant	7.0	16	-[c]
10953	LPS-water phase	7.3	128	46
"	70° C supernatant	7.0	64	23
"	Cell wall preparations	7.5	32	11

[a] reciprocal of the highest dilution displaying at least 1+ HA

[b] as determined by the standard hemolysis assay

[c] none detected

The crude lipid A backbone was tested in the HA and standard hemolysis assays. Both F. nucleatum 4355 and 10953 crude lipid A backbone preparations displayed HA (at a 1:32 dilution) but no measurable hemolysis activity. The HA activity at a 1:32 dilution, was not inhibited by the addition of 100 mM galactose.

DISCUSSION

Hemagglutinating activity has been observed by members of the family Bacteroidaceae. It has been suggested that the attachment mechanisms involved in the hemagglutination may also be important in the colonization and retention of the microorganisms in the oral cavity (64, 72-75). The current studies revealed that following attachment, F. nucleatum whole cells were capable of lysing human erythrocytes under a variety of in vitro test conditions. It appeared that attachment was necessary for the hemolysis to occur as (1) erythrocytes with F. nucleatum attached to their surface, in the absence of non-attached F. nucleatum, were lysed; (2) when F. nucleatum cells were incubated in the presence of galactose containing sugars which have been shown previously to inhibit hemagglutination (75), hemolysis was decreased and (3) the degree of hemolysis increased with increasing microbial whole cell concentrations, also suggesting a specific surface interaction. None of the Bacteroides species tested, although showing hemagglutination, dispayed measurable hemolytic activity. The hemolytic assay was carried out in a non-anaerobic environment so the presence of oxygen sensitive hemolysins cannot be ruled out for both genera.

The hemolytic activity appears to be associated with the LPS of the outer membrane of F. nucleatum as whole cells, cell wall preparations and water phase LPS preparations displayed hemolytic activity. Several investigators have reported that the biologically active portion of the LPS molecule was associated with lipid A (70, 76, 77). The crude lipid A backbone extract, prepared during these investigations, failed to display hemolytic activity. It did however display hemagglutination. Previous studies

have shown that F. nucleatum whole cell hemagglutination was inhibited by galactose (75). In this study a different binding pattern by the lipid A backbond was observed, as galactose did not inhibit the hemagglutination. The fact that the F. nucleatum lipid A backbone preparation was able to bind to the red blood cells without causing lysis, suggested that either the hemolysin was no longer present, having been destroyed by acid hydrolysis, or that a molecule of critical size was a prerequisite for hemolytic activity. Acid hydrolysis has been shown to cleave acid-sensitive linkages and alter the structure of the endotoxin molecule (76). In order for endotoxin to elicit characteristic effects in the mammalian host, a macromolecule of critical size was required (76, 78).

LPS of F. nucleatum is markedly different from that of B. melaninogenicus. The LPS of F. nucleatum has displayed the endotoxin activity typical of other gram-negative bacteria, including Salmonella, E. coli, and Pseudomonas (71, 77). Chemical analyses revealed that the LPS of F. nucleatum contained low concentrations of 2-keto-3-deoxy-octonate and heptose (77, 79-82) whereas that of B. melaninogenicus lacked 2-keto-3-deoxy-octonate, heptose, and typical lipid A (81-83). Vasstrand et al. (84) have reported that the F. nucleatum cell wall uniquely lacks the amino acid diaminopimelic acid, and contains the very rare lanthionine in its place.

The observation that F. nucleatum displays a hemolytic activity associated with its characteristic hemagglutination has opened several avenues of research. The potential of the microorganisms to lyse human erythrocytes in the gingival pocket may release iron containing compounds and other metabolites important to the growth of F. nucleatum and other microorganisms which are seen to increase in number in these polymicrobic infections. Also the potential cytotoxicity of F. nucleatum whole cells or cell wall fragments when attached to human leukocytes, gingival fibroblasts, and crevicular epithelial cells warrants further investigation.

Serologic Characterization of Fusobacterium nucleatum ANUG Isolates with Other F. nucleatum Strains

Introduction

A study was undertaken to compare the hemagglutination activity and reaction of human sera and rabbit anti-F. nucleatum sera with F. nucleatum isolates obtained from humans demonstrating clinically healthy gingiva and various gingival and periodontal disease states. Additional isolates were obtained from dogs and from a Macaca mulatta monkey demonstrating spontaneous chronic periodontitis. The serologic reactions of the F. nucleatum isolates were compared with selected reference strains of F. nucleatum.

Materials and Methods

Cultures and cultural conditions.

F. nucleatum clinical isolates were obtained from the following sources: 1) human adults virtually free of periodontal diseases, 2) human children free of periodontal diseases, 3) human adults with diagnosed chronic periodontitis, 4) human adults with diagnosed acute necrotizing ulcerative gingivitis (ANUG), 5) human adults with diagnosed juvenile periodontitis, 6) two mature dogs, one mixed breed and one golden retriever, with clinical signs of early chronic periodontitis and 7) one Macaca mulatta monkey with spontaneous advanced chronic periodontitis (Table 20). Four typed strains of F. nucleatum (ATCC 10953 and VPI 4355, 5593 and 10197), F. periodoticum (ATCC 33693), F. martiferum (VPI 0501), F. varium (VPI 4123A), and F. nucrophorus (VPI 2891) were also utilized. All clinical isolates were obtained from subgingival plaque samples using a sterile curette (MC 17/18 - Hu Friedy) and immediately streaked onto CVE agar (85), a selective medium for the isolation of F. nucleatum. All samples were incubated at 37° C anaerobically using the BBL anaerobic jar-Gas Pak system (BBL, Cockeysville, Md.) or a Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, Michigan) with an atmosphere of 85% nitrogen, 10% hydrogen and 5% carbon dioxide. After four days of incubation, isolates were identified by colonial morphology (85) and described as type I, a

Table 20

Source of F. nucleatum clinical isolates

Clinical Description	Number of Subjects	Number of Isolates
Human Children free from disease	2	3
Human Adults free from disease	6	7
Human patients with chronic periodontitis	3	3
Human patients with ANUG	10	10
Human patients with juvenile periodontitis	3	6
Canine	2	8
Macaca mulatta	1	11
TOTAL	27	48

2 mm transparent smooth blue colony having an entire edge with a dark blue center or type II, a 1 mm to 2 mm transparent round or irregular blue colony with a speckled appearance. Selected colonies were streaked for isolation on blood agar (BBL) and incubation continued for 48 hr. Each isolate was observed by phase contrast microscopy and Gram stained to verify the typical morphology of Fusobacterium. Only isolates which demonstrated Gram negative staining with the morphology of long rods with pointed ends and failing to grow aerobically on blood agar were selected for further study. Biochemical reactions were determined using the API 20A system (Analytab Products, Plainville, N. Y.). Isolates were then transferred to a modified tryptone medium (64) and incubated as before. Cells were harvested by centrifugation at 10,000 x g for 10 min at 4^{o} C, washed three times in 0.01 M phosphate buffer containing 0.15 M NaCl (PBS), pH 7.2 and resuspended in PBS to a 10% suspension (1:10 dilution of packed whole cells after centrifugation at 2,000 x g for 10 min).

Whole cell suspensions of these isolates were sonicated using 8 bursts of 30 sec each in a dry ice-alcohol bath with a Heat Systems Sonicator (Plainville, N. Y.) at a microtip setting of 7. Greater than 95% of the cells were lysed as observed by phase contract microscopy. This was designated as the sonicated preparation (SP).

Double diffusion in agar and immunoelectrophoresis.

SP of selected isolates and typed strains of Fusobacterium nucleatum were reacted by double diffusion (86) in 1% agarose in 0.01 M PBS, pH 7.2 with undiluted rabbit anti-F. nucleatum 10953 or 10197 serum (64). Glass slides overlaid with 3.0 ml of the 1.0% buffered agar gel was used for immunoelectrophoresis. The slides containing SP were electrophoresed in Verond buffer, pH 8.6 (69). Reactions were incubated at 4^{o} C in a humidor and were observed after 24 hr. Protein content of SP as determined by Lowry et al. (87) was aproximately 400 ug/ml).

Serological evaluation by ELISA.

Selected isolates were reacted by an enzyme-linked immunosorbent assay (ELISA)

with the following sera: 1) rabbit anti-F. nucleatum 10953 or 10197 serum, 2) normal rabbit serum, 3) human sera previously shown to be reactive to 10953, 10197 and 4355 and 4) serum obtained from the Macaca mulatta demonstrating spontaneous chronic periodontitis. A modification of the enzyme-linked immunosorbent assay (ELISA) was utilized (88). Two hundred microliters of a 1:10 dilution of the previously described whole cell suspensions of F. nucleatum in a 60 mM carbonate buffer, pH 9.6, were added to each well of a microtiter plate (Dynatech Laboratories, Inc., Alexandria, VA) and incubated at 37° C for 3 hr followed by refrigeration overnight. Peripheral rows were not utilized. The plate was washed five times with 0.01 M PBS containing 1% BSA and 0.05% tween 20. This was followed by a 3 hr incubation at 37° C with 200 ul of a 1% BSA solution in 60 mM carbonate buffer in order to assure complete coverage of all binding sites in each well. The plate was then washed five times with PBS/tween 20 with 1% BSA. One hundred microliters of serial two fold dilutions in 0.01 M PBS with 0.05% tween 20, pH 7.2, of the sera were added and incubated at 37° C for 30 min. The plates were washed as before and 100 ul of a 1:200 dilution in PBS of peroxidase labeled IgG fraction of goat anti-rabbit , and heavy chain serum or peroxidase labeled goat anti-Rhesus gamma/globulin serum (Cappel Laboratories, Inc., Cochranville, Pa.) were added and the plate incubated again at 37° C for 30 min. After again washing the plates five times, 100 ul of the enzyme substrate (1 ml of 1% w/v O-phenylene-diamine in methanol plus 99 ml distilled H_20 and 0.1 ml 3% H_20_2) were added. The plates were incubated in the dark for 30 min at room temperature, the reaction was stopped by adding 20 ul of 8N H_2SO_4 and reactions were determined colormetrically at 490 nm with a Microelisa Reader (Dynatech Laboratories, Inc.). To determine the optimal concentration of antigen and peroxidase labeled goat antiserum, a dual titration of doubling dilutions of antigen (0.25% to 10% whole cell suspensions) against serial 1:5 fold dilutions of conjugate was performed. Analysis of the protein content of the 1% whole cell suspensions (1:10 dilution of the 10% suspension) was determined to be 40% ug/ml by

the technique described by Lowry et al. (87).

Hemagglutination (HA)

The HA assay was performed by the method of Falkler and Hawley (64). Briefly, serial two-fold dilutions of 50 l of 10% whole cell suspensions of selected isolates of F. nucleatum were made in microtitration multi-well plates (Linbro Scientific, Inc., Hamden, Conn.) with 0.01 M PBS, pH 7.2. This was followed by the addition of 25 l of PBS, pH 7.2 and 25 l of a 1.25% suspension of sheep red blood cells (RBCs). Plates were mixed for 20 sec on a Micro-Shaker (Cooke Laboratory Products, Alexandria, Va.) then incubated for 30 min at 37° C followed by 1 hr at 4° C. Titers were determined visually as the reciprocal of the highest dilution demonstrating a 2+ hemagglutination. Inhibition of HA was determined as above except for the addition of 25 l of 50 mM of D-galactose in place of PBS and incubation for 30 min at 37° C prior to adding the sheep RBCs.

Results

Isolation of F. nucleatum

F. nucleatum isolates were obtained on CVE agar plates from all plaque samples. The human isolates demonstrated both type I and type II colonial morphology as did isolates from the Macaca mulatta monkey. All canine isolates showed only type I colonial morphology. All isolates regardless of colony type showed identical results with the API 20A system (a positive indole response with all other reactions negative).

Serological evaluation by immunodiffusion.

SP of F. nucleatum strains 10953, 10197 and 4355 were reacted with rabbit anti-F. nucleatum 10953 and 10197 sera by double diffusion in agar. Several precipitin lines including lines of identity were observed for all typed strains. SP of sixteen separate isolates from various human oral disease states (including ANUG isolates), three typed strains of F. nucleatum and F. periodonticum were also reacted with rabbit anti-F. nucleatum 10953 and 10197 sera. Lines of identity were evident between all sixteen clinical isolates of F. nucleatum, the three typed strains and F. periodonticum. The

eleven Macaca mulatta monkey isolates, eight canine isolates, three human isolates and strain 10953 were also reacted with the rabbit anti-F. nucleatum 10953 serum. Of all nineteen of the animal isolates tested, only two of the Macaca mulatta strains shared lines of identity with the human isolates and 10953.

SP of 10197, 10953, ANUG isolates and F. periodonticum also displayed lines of identity when reacted with Guinea pig anti-F. nucleatum 10197 and 10953 sera. As with the rabbit sera, pre-bleed Guinea pig serum did not show any precipitin lines with the SP of any strains. SP of F. nucleatum (4355, 5593, 10197 and 10953), F. mortiferum, F. varium, F. necrophorus, F. periodonticum and several F. nucleatum ANUG isolates were reacted in immunodiffusion with rabbit anti-F. nucleatum 10197 and 10953 sera. As before all of the F. nucleatum strains and F. periodonticum shared lines of identity however, no lines of identify were observed between the F. nucleatum or F. periodonticum strains and any of the other Fusobacterium species tested. Similar lines of identity were observed when Guinea pig antisera to F. nucleatum 10197 and 10953 were reacted with the various strains.

Immunoelectrophoresis experiments confirmed what had been observed with the immunodiffusion testing in that F. nucleatum strains from all human sources (typed and clinical isolates) shared several antigens with each other and F. periodonticum, however no precipitin bands were observed with any of the other Fusobacterium species with the exception of a small diffuse line located near the antigen well but slightly towards the anode observed when rabbit anti-F.nucleatum 10953 serum was reacted with F. necrophorus.

Eleven human isolates of F. nucleatum were reacted by ELISA with rabbit anti-F. nucleatum 10197 or 10953 serum and with normal rabbit serum. As can be observed in Table 21, similar titers for all isolates were obtained with both hyperimmune sera which displayed substantially higher titers than those observed with normal rabbit serum.

Twenty-three human clinical isolates of F. nucleatum were reacted by ELISA with

Table 21

Elisa titers obtained with human isolates of *F. nucleatum* and anti-*F. nucleatum* and pre-immune sera.

Patient Classification	Sera: Rabbit anti-*F. nucleatum* 10197 mean titer	Sera: Rabbit anti-*F. nucleatum* 10953 mean titer	Sera: Normal rabbit mean titer
Normal adult (6)[a]	6[b]	6	3
Patients with acute necrotizing Ulcerative Gingivitis (5)	6	6	3

[a] number of patients providing isolates of *F. nucleatum*

[b] highest dilution giving an OD reading of greater than 0.15 at 490 nm after transforming the results from geometric progressions to arithmetic progressions (1:16=1, 1:32=2, 1:64=3, etc.

a human serum previously shown to be reactive with F. nucleatum. The titers that were obtained are shown in Table 22. Irregardless of the source of the F. nucleatum isolates, a similar range of antibody activity was detected with the human serum.

The results obtained when ten Macaca mulatta isolates, eight canine isolates, four human isolates and 10953 were reacted with 1) human sera previously shown to be reactive with F. nucleatum, 2) serum from the Macaca mulatta with spontaneous periodontal disease and rabbit anti-F. nucleatum can be seen in Table 23. All sera showed antibody reactivity to the human (including 10953) and Macaca mulatta strains but not to the canine isolates.

Hemagglutination (HA)

Eleven Macaca mulatta isolates, eight canine isolates, three human isolates and srain 10953 were tested for HA activity and with the addition of 50 mM D-galactose, for inhibition of HA. All canine isolates consistently failed to shown any HA of sheep RBCs (Table 24). Some of the Macaca mulatta isolates and all of the human isolates showed varying degrees of HA activity. All isolates which demonstrated HA activity also showed a decrease in HA when preincubated with 50 mM D-galactose prior to HA testing.

Table 22

Elisa titers obtained with human isolates of

F. nucleatum and a human sera

Patient Classification		mean titer[b]
Normal adult	7[a]	5.7 (5-6)[c]
Normal child	3	6[d]
Acute necrotizing ulcerative gingivitis	5	5.6 (4-6)
Chronic periodontitis	2	7[c]
Juvenile periodontitis	5	6.8 (6-8)

[a] number of patients providing isolates of F. nucleatum and number of isolates tested

[b] after transforming the results from geometric progressions to arithmetic progressions (1:10=1, 1:20=2, 1:40=3, etc.)

[c] range of titers

[d] all titers were the same within this group

Table 23

Elisa titers obtained with clinical and typed strain isolates of *F. nucleatum* with selected sera.

F. nucleatum isolates		Human	Rabbit anti-*Macaca mulatta*	*F. nucleatum* 10953 Serum
Macaca mulatta	10[a]	2.9[b] (3-4)[c]	4.7 (4-5)	5.7 (5-6)
Canine	8	1	1	1
Human	4	5.5 (5-6)	4[d]	7
10593	1	6	4	8

[a] number of isolates from each source

[b] after transforming the results from geometric progressions to arithmetic progressions (1:10=1, 1:20=2, 1:40=3, etc.)

[c] range of titers

[d] all titers were the same

Table 24

Hemagglutination obtained from clinical isolates of F. nucleatum and 10953.

Isolates		Hemagglutination titers	Hemagglutination titers after galactose inhibition
Macaca mulatta	11	3.3[b] (1-8)[c]	.91 (1-1)
Canine	8	[d]	[d]
Human	3	6.3 (4-8)	4.3 (3-6)
Typed strain 10953	1	11[e]	9[e]

[a] number of isolates from each source

[b] highest dilution displaying at least a 2+ HA after transforming the results from geometric progressions to arithmetic progressions (1:2=1, 1:4=2, 1:8=3, etc.)

[c] range of titers

[d] hemagglutination was not observed at the lowest dilution used - 1:2

[e] all titers were the same

Discussion

F. nucleatum has been implicated in various human oral disease states (89, 90). Recent information suggests genetic heterogeneity between F. nucleatum isolates from different sites in the same mouth (Y. Selin and J. L. Johnson, J. Dent. Res. 1981, 60, special issue A, p. 15). The results of this study support the concept that human F. nucleatum isolates share antigenic determinants. This concept must be taken into consideration when using immunodiagnostic methodologies or considering the contribution of F. nucleatum to the immunopathology of different disease states.

The increase in numbers of F. nucleatum which has been reported in isolation studies of oral lesions (89-94) may reflect an ecological change resulting from an environment more conducive to the colonization and growth of these anaerobic microorganisms. To colonize in the complex ecosystem of the periodontal pocket and resist the natural defense and cleansing mechanisms of the host, it would be advantageous for F. nucleatum organisms to have a means of binding to human cell surfaces or to other microorganisms or their products. Oral F. nucleatum strains show a characteristic HA (64) and attach to human oral epithelial cells, gingival fibroblast cells, and leukocytes (95). This involves a glycoprotein on the surface of the bacterium binding to a galactose-containing moiety on the cell surface via a Ca^{2+} dependent reaction (75). The HA-active moiety of F. nucleatum contains or is very near to a major antigenic determinant, as rabbit anti-F. nucleatum serum inhibits HA activity, the hypothesis being that antibody, upon reaction with the antigenic determinants with the human isolates also did not show HA with the sheep RBCs. It would be of interest to determine whether these canine isolates would hemagglutinate dog RBCs or bind to canine buccal epithelial cells. Possibly, canine F. nucleatum isolates have adapted to utilize a different means of attachment than the one observed from human F. nucleatum strains.

Recently, a new Fusobacterium species was isolated from monkey dental plaque and designated Fusobacterium simiae (96). This organism was described as being

morphologically similar to the M. mulatta monkey isolates described here, but they differed biochemically (fermentation of glucose was positive in F. simiae monkey isolates and negative in M. mulatta monkey isolates).

Efforts have been directed in the past toward developing a suitable animal model system for evaluating the microbiological aspects of the development and progression of periodontal diseases (91, 97-99). The results presented here suggest that the antigenic characteristics of F. nucleatum strains isolated from animals may significantly differ from those isolated from humans. What role these results may play in the significance of F. nucleatum in the etiology of periodontal diseases in different host systems is unclear. If, however, an immunological basis of disease is considered, the differences in antigenic determinants in F. nucleatum isolates from different species of animals create difficulty in extrapolating results from animal studies to the naturally occurring periodontal diseases in humans.

Serological Reaction of Clinical Isolates and Typed Strains of the Family Bacteroidaceae

Introduction

Improved techniques for the cultivation of anaerobic bacteria have resulted in a heightened awareness of the importance of these pathogens in ANUG and other clinical infections. Currently interest exists for the development of serological techniques for presumptive identification of anaerobes since rapid identification of these organisms is important in decisions concerning treatment. B. gingivalis and B. fragilis are anaerobic organisms frequently associated with oral or non-oral lesions whereas B. melaninogenicus has been isolated from both oral and non-oral lesions. This study reports the use of radial immunodiffusion to examine the reaction of rabbit antisera to B. gingivalis. B. fragilis, and B. melaninogenicus ssp. melaninogenicus (B. mel mel) with clinical isolates and typed strains of the family Bacteroidaceae.

Materials and Methods

Bacterial Strains and Media. The organisms listed in Table 25 were used in this study. B. gingivalis and B. melaninogenicus ssp. intermedius srains were cultured from subgingival plaque of patients with chronic periodontitis and identified by Dr. Carol Spiegel in the Department of Microbiology, University of Maryland Dental School. B. mel mel, Capnocytophaga (Bacteroides) ochraceus (27872), and two strains of Fusobacterium nucleatum (10197 and 10953) were obtained from the American Type Culture Collection (ATCC), Rockville, Md. F. nucleatum 4355 was obtained from the Virginia Polytechnic Institute (VPI), Blacksburg, Virginia. B. asaccharolyticus strains were isolated from patients and identified at the Baltimore Cancer Research Program, University of Maryland Hospital, Baltimore, Md. B. fragilis (JS-2), a clinical isolate, was obtained from Dr. Jean Setterstrom at the Walter Reed Army Institute of Dental Research, Washington, D. C. All other strains of Bacteroides were obtained as clinical isolates by Dr. Sharon Hansen at the Veterans Administration, Baltimore, Md.

All Bacteroides strains were grown in Brain Heart Infusion broth (BBL, Cockeysville, Md.) supplemented with hemin (Sigma Chemical Co., St. Louis, Mo.) 5 ug/ml, and menadione (Sigma), 1 ug/ml. Capnocytophaga and Fusobacterium strains were grown in modified tryptone medium containing 1% tryptone (Difco, Detroit, Mich.), 1% yeast extract (BBL), 0.2% dextrose, 0.5% thioglycollate, 0.125% K_2HPO_4, and 0.125% $MgSO_4.7H_20$. All cultures were incubated at 37° C in either a Coy Anaerobic Chamber (Coy Laboratory Products, Inc., Ann Arbor, MI) or in a BBL anaerobe jar with GasPak for 48-72 hr.

Antisera Production Bacteria from broth cultures were harvested by centrifugation at 23,500 x g for 30 minutes and washed x3 in 0.01 M phosphate 0.15 M NaCl buffer, pH 7.2 (PBS). The organisms were resuspended in PBS to a 10% suspension (V/V) or to an optical density (O.D.) of 0.6 using a Klett-Summerson photoelectric colorimeter (Klett Manufacturing, Inc., New York, N.Y.). Sonicated antigen preparations were made by

ultrasonication of 10% bacterial suspensions in a dry ice-alcohol bath using a Branson Sonifier (Branson Instruments, Inc., Stanford, Conn.) at 6 amps for 8 bursts of 30 sec each. The sonicated suspensions were then centrifuged at 23,500 x g for 30 min and the supernatant fluids (SSF) removed. The SSF were sterilized using a 0.45 um filter (Millipore) and the protein concentrations determined by the method of Lowry et al. (87) using bovine serum albumin fraction V (Sigma) as a standard. Emulsions were prepared by using equal volumes of Freund complete adjuvant (Difco) and filter sterilized SSF (2.0 mg protein/ml emulsion). Each week for four weeks the SSF-adjuvant emulsion (0.25 ml) was injected subcutaneously at four sites in the backs of male Swiss albino rabbits (2-4 kg). Whole cell suspensions (O.D. of 0.6) were also used for intravenous injection of other Swiss albino rabbits according to the following schedule: 0.1 ml on day 1, 0.2 ml on day 2, and 0.3 ml on day 7, followed by intraperitoneal injections of 1.0 ml at 2 weeks and 5 weeks. One to three weeks after the final inoculation, rabbits were exsanguinated via the marginal ear vein. Clotted blood was centrifuged at 600 x g for 10 min, the serum removed and merthiolate (1:10,000) added. The sera were stored in 1-2 ml volumes at -20° C until ready for use in serologic testing.

Antigen Preparation for Radial Immunodiffusion (RID) Bacteria from broth cultures were harvested at 600 x g, washed in PBS, and resuspended in PBS to a concentration of 10% (V/V). While immersed in a dry ice-alcohol bath, these suspensions were treated by sonication with a Branson sonifier at 6 amps for 8 bursts of 30 sec each. The sonicated preparations were stored at -20° C until ready for use in serological testing.

An antigen preparation of B. gingivalis (CS-43) resulting from treatment of whole cells with acid was also used. Cells from 1.0 ml of a 10% (V/V) suspension in PBS were sedimented by centrifugation at 600 x g, the supernatant fluid (PBS) discarded, and the cells resuspended in 0.4 ml of 1N HCl. After incubation at 37° C for 30 min, the suspension was neutralized with 1N NaOH, and PBS added to produce a final volume of

1.0 ml. This procedure was repeated for acid-heat extraction of B. gingivalis antigens except incubation at 37° C was replaced by autoclaving at 121° C for 15 min. A heat extraction antigen preparation of B. gingivalis was prepared by autoclaving (121° C for 15 min) 1.0 ml of a 10% (V/V) suspension of bacteria in PBS. An antigen preparation was also prepared by rapidly freezing a 10% suspension of B. gingivalis in PBS in a dry ice-ethanol bath followed by thawing the suspension in a 56° C water bath. The procedure was repeated a total of ten times.

Adsorption Studies. Each antiserum was adsorbed with homologous whole cell suspensions. Four ml of a 10% (V/V) suspension of bacterial cells in PBS were centrifuged at 23,500 x g for 30 min and the supernatant fluid discarded. The sediment was resuspended in 3.6 ml of homolgous antiserum. The resulting suspension was incubated at 37° C for 30 min with occasional mixing. The cells were then removed by centrifugation at 23,500 x g for 30 min. The adsorption procedure was repeated a total of 4 times. During the final adsorption, the antiserum-antigen suspensions were incubated at 4° C overnight. Following removal of bacterial cells by centrifugation, adsorbed antisera were stored at -20° C until used in serological studies.

RID Technique. A 1% agarose (Calbiochem, Los Angeles, Ca.) solution was prepared in a glycine buffer (pH 7.0) containing 7.52 g/L glycine in 0.04 M EDTA with 1 g/L of merthiolate. The temperature of the agarose solution was stabilized at 52° C in a water bath. A predetermined volume of antiserum at room temperature was mixed with the agarose solution to achieve a final volume of 4 ml. Various bleedings and dilutions of the anti-B. gingivalis, anti-B. fragilis and anti-B. mel mel sera were tested to determine the strongest reactions with homolgous antigen preparations while allowing the greatest conservation of antiserum. The optimal dilution of the selected anti-B. gingivalis serum was determined to be 1:4, anti-B. fragilis serum - 1:2, and anti-B. mel mel - 1:4. The mixture was then immediately poured into an immunodiffusion plate (Hyland, Costa Mesa, Ca.). After storing the plates at 4° C overnight, wells (3 mm in dimaeter) were

prepared in the agar and filled with the bacterial antigen preparations. The plates were incubated in a humidor at 37° C and observed at 48 hr for the presence of precipitin rings. The diameter of the precipitin rings was measured in mm by using a Hyland Precision Viewer (Hyland, Costs Mesa, Ca.). Precipitin rings 4.0 mm were recoreded as positive.

Results

The RID reaction of antiserum with homologous antigen demonstrated that SSF produced the greatest number of precipitin rings when compared with antigen preparations produced by autoclaving, acid extraction, a combination of autoclaving and acid extraction, or "freeze and thaw" treatment of bacterial suspensions. These results determined SSF to be the antigen preparation of choice for all subsequent experiments.

Anti-B. gingivalis serum was reacted with 39 strains of various Bacteroides species, two strains of F. nucleatum and one strain of C. ochraceus. The SSF of three strains of B. gingivalis produced from two to four precipitin rings when reacted with anti-B. gingivalis serum. These precipitin lines also varied in intensity and in the diameter of the rings. One strain of B. thetaiotaomicron produced one weak yet distinct precipitin ring (5.3 mm in diameter) whereas none of the other five strains of B. thetaiotaomicro showed a positive reaction with anti-B. gingivalis serum. None of the antigen preparations of the other 35 strains reacted when tested with the anti-B. gingivalis serum (Table 25).

Anti-B. fragilis serum showed a range of reactivity with the various bacterial antigen preparations (Table 25). All strains of B. distasonis and B. ovatus demonstrated 1 to 4 precipitin rings. Five of the six B. fragilis strains tested, five of six B. thetaiotaomicron strains, and two to three B. uniformis strains showed positive reactions having 1-3 precipitin bands. None of the three B. vulgatus strains demonstrated a precipitin ring. The SSF of C. ochraceus produced one strong ring 5.2 mm in diameter when reacted with anti-B. fragilis serum. Each of two strains of F. nucleatum produced

Table 25

Reaction of sonicated antigen preparations

with rabbit anti-*Bacteroides* species sera

Species	Strain used for RID	Strain used to prepare Antiserum CS-43	JS-2	25845
Bacteroides gingivalis		CS-41		2[a] - 2
CS-43	3	-	-	
CS-47	4	-	1	
B. asaccharolyticus	MH678	-[b]	-	2
MH697	-	-	-	
MH803	-	-	-	
B. mel. mel.	25845	-	1	3
B. mel. ssp. *intermedius*		CS-28		- N.D.[c] -
B. loescheii	15930	-	N.D.	1
B. intermedius	15032	-	N.D.	1
B. distasonis	F232	-	1	-
F193	-	2	-	
F244	-	4	-	
F315	-	2	-	
F73	-	2	-	
F274	-	1	-	
B. fragilis	JS2	-	2	-
F379	-	1	-	
F377	-	-	1	
F378	-	2	-	
F382	-	2	-	
F381	-	3	-	
B. ovatus	F258	-	3	-
U55	-	1	-	
F495	-	3	-	
F257	-	1	-	
F338	-	2	-	

Table 25 (con't.)

B. thetaiotaomicron	F135	-	2	-		
F224	-	2	-			
F121	1	-	-			
F266	-	1	-			
F137	-	1	-			
F225	-	1	-			
B. uniformis	F451	-	-	-		
F373	-	1	-			
F367	-	1	-			
B. vulgatus	F424	-	-	-		
F261	-	-	-			
F500	-	-	-			
Capnocythophaga ochraceus		27872		-	1	-
Fusobacterium nucleatum		10197		-	1	-
10953	-	1	-			
4355	N.D.	N.D.	-			

[a] number of distinct precipitin rings 4.0 mm in diameter

[b] no precipitin rings observed

[c] not determined

a single precipitin ring (about 4 mm diameter) when tested with the anti-B. fragilis serum. A strain of B. mel mel produced a single precipitin ring (5.0 mm in diameter) whereas none of the other nine strains of black pigmented Bacteroides showed reactivity.

Anti-B. mel mel serum when tested with its homologous antigen produced 3 precipitin rings, but only one ring was formed with two other strains of B. mel mel. The reaction of the antiserum was inconsistent with other species of black pigmented Bacteroides, producing reactions with 2 of 3 B. gingivalis strains, 1 of 3 B. asaccharolyticus strains and no reaction with the single strain of B. melaninogenicus ssp. intermedius tested. Of all 33 other strains of Bacteroidaceae only one strain of B. fragilis produced a precipitin ring when reacted with anti-B. mel mel serum.

After antisera were adsorbed with whole cell suspensions of homologous organisms, precipitin reactions were either reduced or removed. Adsorbed anti-B. gingivalis serum produced only a single, weak precipitin ring when reacted with two strains of B. gingivalis and showed no reaction with the third strain. The adsorbed antiserum was also not reactive with the previously reactive strain of B. thetaiotaomicron. None of the other antigen preparations which previously displayed a positive reaction with anti-B. fragilis serum displayed a reaction with the adsorbed antiserum.

Discussion

When used in RID, anti-B. gingivalis serum exhibited strong reactions with strains of homologous species and also reacted with only one other species tested, B. thetaiotaomicron. This reactivity was removed upon adsorption with B. gingivalis suggesting these two organisms have similar antigenic determinants. This is the first report of a sharing of antigens between B. gingivalis and other microorganisms as other reports have demonstrated the antigenic specificity of B. gingivalis (100, 101).

Anti-B. fragilis serum demonstrated reactions with every Bacteroides species tested with the exception of B. vulgatus, B. asaccharolyticus, and B. gingivalis. These precipitin bands were removed after the adsorption of anti-B. fragilis serum by B. fragilis

suggesting that the Bacteroides species which displayed a positive reaction shared at least one antigen with B. fragilis. Although some studies have reported antigens specific for the B. fragilis species (102-108), or described multiple serotypes (103, 109, 110) other published data report anti-B. fragilis group (108, 110-117) or species of Bacteroides not of the B. fragilis group (111, 118, 119). This current study suggests that F. nucleatum and C. ochraceus share antigenic determinants with B. fragilis . Since precipitin reactions were removed by adsorption of the anti-B. fragilis serum by B. fragilis, our data suggests the presence of B. fragilis antigenic determinants which may be similar to those found in other members of the Bacteroidaceae.

Anti-B. mel mel serum reacted not only with homologous species, but also showed cross reactions occurring with B. gingivalis and B. asaccharolyticus. In other studies oral strains of asaccharolytic black pigmented Bacteroides were not reactive with antiserum to B. mel mel (100, 101). It is possible the reaction was related to the "Bacteroidaceae cross-reacting antigen" recently reported to have been extracted from several strains of B. melaninogenicus (subspecies not identifed), B. oralis, B. bivus, and several species of Fusobacterium (120). Anti-B. mel mel serum was found to react with only one strain of B. fragilis. Other studies have shown conflicting data concerning reactions between B. mel mel and organisms of the B. fragilis group (120, 121).

The method of antigen preparation was important in obtaining a detectable precipitin reaction. In this study the most effective method was ultrasonication of a cell suspension. Repeated freezing and thawing of cells was also effective but to a lesser degree than the sonicated preparation. Heat and/or cold acid extraction were inadequate. Similar results were noted by Reed et al. (101) who found that B. gingivalis antigen prepared by ultrasonication in a cold environment produced more precipitin bands when reacted with homologous antiserum than did antigens prepared by autoclaving or alkali extraction.

In summary the data suggests RID can be used for the identification of B.

gingivalis. With the exception of a single strain of B. fragilis, anti-B. mel mel serum could be used to detect all species of the black-pigmenting Bacteroides tested indicating this antiserum may be useful in RID for early identification of this group of bacteria. The sharing of antigenic determinants by B. fragilis with other species indicates the difficulties of identification of B. fragilis by serological techniques such as RID. It would appear that precise serological identification of B. fragilis must await the characterization of specific antigenic determinants for serogroups and the production of monoclonal antibodies to these determinants. The presence of shared antigens by species of the Bacteroides must be considered when studying immune responses to B. fragilis or B. mel mel.

Reaction of Serum from ANUG Patients with *Fusobacterium* and *Bacteroides* Strains

In an attempt to detect antibody in the sera of ANUG patients with *Bacteroides* and *Fusobacterium* strains enzyme linked immunosorbent assays were established. Sera from ANUG patients and age and sex matched healthy individuals were reacted with *Fusobacterium nucleatum* and *Bacteroides* strains isolated from ANUG patients, chronic periodontitis patients, non-oral abscesses and healthy gingival sulci. Experiments were conducted to measure reactive isotypes of IgA, IgM and IgG and in other experiments IgG only.

MATERIALS AND METHODS

Micro-Enzyme-Linked Immunosorbent Assay (ELISA)

Briefly, 200 l of a 1% *Fusobacterium* or *Bacteroides* whole cell suspensions (grown as previously described in HA methodology) in .06 M carbonate buffer, pH 9.6, were added to each well of a microtiter multi-well plate (outside rows of wells not used) and incubated at 37° C for 3 h followed by refrigeration overnight. The plate was washed three times with PBS containing 0.05% Tween 20 and 1% BSA. Then 200 l of a 1% BSA solution in 0.06 M carbonate buffer, pH 9.6, were added to the wells and incubation allowed to proceed for 3 h at 37° C in order to tie up all non-antigen coated binding sites. The tray was then rinsed five times with PBS/Tween 20 with a 1% BSA. Serial two fold dilutions of the sera (50 l) in PBS were added to each well and the plates incubated at 37° C for 30 min. The plates were washed as before and *Fusobacterium* and *Bacteroides* absorbed peroxidase labeled IgG fraction of goat anti-human , or heavy chain serum (Miles Laboratories) diluted 1:200 in PBS, was added to each well (50 l) and incubated for 1.5 h at room temperature. After again washing the plates, 50 l of the enzyme substrate (1 ml of 1% w/v O-phenylene-diamine in methanol + 99 ml distilled H_2O_2) were added. After 1.5 h of incubation in darkness at room temperature the

reaction was stopped by the addition of 8 N H_2SO_4 (25 l) and the color was read at 490 nm using a Chromoscan spectrometer.

To determine the amount of Bacteroides and Fusobacterium (antigen) required to coat the wells of the microtiter plate and to determine a dilution at which the peroxidase labeled goat antisera should be used, a dual titration of doubling dilutions of antigen (0.25 to 10% whole cell suspensions) against serial 1:5 fold dilutions of conjugate was performed. An excess of reactive sera (1 in 100 dilution) was added to each well. A 1% suspension of Bacteroides and Fusobacterium strains and a 1:200 dilution of conjugate were found to be concentrations which would give an optimum yellow color and were the standard dilutions used for all subsequent experiments. All serologic tests were performed in duplicate and when more than a 0.15 O.D. difference was observed, testing of the serum was repeated. Controls included the reaction of sensitized wells with saline replacing human serum in the assay and reacting substract with conjugate to check on enzyme activity.

RESULTS

Five sera from ANUG patients and age matched sera from individuals with healthy mouths were reacted with 18 strains of F. nucleatum and 12 Bacteroides strains. The microorganisms represented ATCC and VPI strains, clinical isolates and isolates from ANUG lesions.

When the five sera were reacted with the 18 F. nucleatum isolates in an ELISA which would detect IgG, IgA and IgM it was observed that the sera displayed similar degrees of activity irregardless of the F. nucleatum strain used as the antigen source (Table 26). It thus appears all of the F. nucleatum strains tested displayed shared antigenic determinants. It was also observed that there were differences in the activity of the sera, some showing significant differences in the amount of antibody reactive with the F. nucleatum strains. When IgG was detected in the ANUG sera and age matched sera from individuals with healthy oral cavities (Table 27) it was observed that there were no differences in the mean antibody activity to the 18 strains in the two groups and in three of the five sera pairs, a slightly higher level of antibody was detected in the sera from the healthy individuals. Again it appeared that IgG was present in each sera to all of the organisms tested and that similar levels of antibody reactive with each organism was detected.

Although a somewhat higher level of antibody was found reactive with the Bacteroides strains the data paralleled what was observed with the F. nucleatum testing. It appears that the B. gingivalis strains isolated from the ANUG patients share antigens with other B. gingivalis clinical isolates (Table 28) and that no differences were observed between the reactivities of the patient and age matched control sera (Table 29). It was of interest that the levels of antibody in both groups reactive to the non-oral B. fragilis and B. ovatus strains were lower than those observed for the oral strains (Tables 30 & 31).

This data suggests that:

(1) Fusobacterium as well as Bacteroides isolates from ANUG lesions each share antigenic determinants.

(2) A single serum sample taken during an ANUG episode when reacted with Fusobacterium or Bacteroides strains appears not to be useful in the diagnosis of the disease.

Table 26

ELISA Measuring IgG, IgA & IgM

F. nucleatum strain	ANUG Patient Serum Source XII	XIV	XV	XVI	XIX
4355	528[a]	435	861	330	997
Lai 716	508	1169	463	954	
10953	1028	725	1166	560	1223
CD_3 495	243	930	140	720	
VIII A_{14}	506	271	581	207	681
X A_{12}	444	323	827	105	751
$XIII_2$	397	206	629	12	568
WAF 460	247	746	126	690	
VI A_1	678	307	1215	162	872
$XVII_8$	896	610	1022	565	910
XVI_2	705	768	983	505	1059
DS_1 444	225	704	111	744	
XII_3	520	366	839	199	1010
CD_2 429	346	836	66	721	
MR_3 462	345	888	236	738	
VI A_2	571	433	1131	127	761
VIII A_{13}	289	220	574	33	612
10197	560	490	1029	214	860
Mean =	535	393	896	232	826
S. E. =	121	89	202	52	186

[a] optical density at 490 nm - average of duplicates at a 1:20 dilution

Significant difference (by S. D.) between 1) serum XV and XIV, XVI; 2) serum XIX and XIV, XVI

Table 27

ELISA Measuring IgG

F. nucleatum strain	XII	Control	XIV	Control		XV	Control	XVI	Control	XIX	Control
4355	725[a]	1383	--[b]	1645	1240	746	378	1999	1363	1311	
Lai	587	410	905	1635	1373	1005	708	773	1216	762	
10953	588	734	1153	1600	1086	1003	697	629	1488	798	
CD 3	162	227	589	1072	634	662	399	453	1043	538	
VIII A14		292	493	--	995	701	1013	423	707	1032	360
X A12	221	329	955	480	741	515	654	437	1122	480	
XII 2	331	284	1481	392	392	650	450	539	1078	366	
WAF	412	221	1104	547	827	545	788	616	1192	563	
VI A	753	308	1072	732	1589	557	881	787	1035	990	
XVII 8	811	636	1457	748	1091	830	1216	689	1399	554	
XVI 2	592	760	1319	1053	942	1294	--	494	321	667	
DS 1	680	351	492	939	795	1025	--	498	1063	559	
XII 3	464	559	529	1048	1122	865	1083	614	1021	697	
CD 2	296	308	383	946	774	600	425	560	1088	398	
MR 3	509	966	486	1228	1210	568	843	427	1085	782	
VI A2	497	879	--	1129	1534	755	792	619	1253	761	
XVIII AB		682	234	478	1544	1012	801	746	932	995	389
10197	574	731	--	1375	811	776	1054	748	1054	320	
Mean	509.8	545.2	689.1	1061.6	993.0	789.4	640.9	695.6	1082.1	627.5	
S.E.	120.2	128.6	162.5	250.4	234.2	186.2	151.2	164.1	255.2	148.0	

[a] optical density at 490 nm
[b] no activity

Table 28

ELISA Measuring IgG, IgA and IgM

B. gingivalis strain	ANUG Patient Serum Source					
	XII	XIV	XV	XVI	XIX	X/SD
XV 6 738[a]	574	1448	672	1501	98.6/449.6	
XII 8	627	709	1009	788	982	823.0/167.7
CS41 723	510	1221	283	1614	870.2/541/4	
CS43 1194	921	1590	609	1596	1182.0/928.5	
CS44 1093	823	1705	582	1663	1161.2/503.4	
Mean =	863.0	707.4	1394.6	586.8	1471.2	
S.D. =	239.5	170.1	281.1	187.4	279.7	

[a] optical density at 490 nm - average of duplicates

Table 29

ELISA Measuring IgG

B. gingivalis strain	XII	Control	XIV	Control	XV	Control	XVI	Control	XIX	Control	ANUG X/SD	Control X/SD
XV6	1349[a]	1730	1622	1223	1690	1736	1613	1745	1680	1496	1590.8/139.4	1586.0/228.2
XII8	406	489	--[b]	608	675	175	373	861	1055	399	501.8/391.7	334.8/244.0
CS41	1407	1802	311	1133	1390	1660	1235	1738	1620	406	1192.6/511.5	1347.8/589.3
CS43	1640	1538	1643	1670	1551	1697	1011	1805	1738	419	1515.6/290.3	1427.8/566.4
CS44	1675	1679	1551	--	1432	1660	588	1941	1392	710	1327.6/428.0	1198.0/817.0
Mean	1295.4	1447.6	1025.4	926.8	1347.6	1385.6	964.0	1618.0	1497.0	688.0		
S.D.	517.0	544.5	802.4	640.9	393.7	677.4	496.9	431.0	279.8	470.0		

[a] optical density at 490 nm
[b] no activity

Table 30

ELISA Measuring IgG, IGA and IgM

Bacteroides strain	ANUG Patient Serum Source XII	XIV	XV	XVI	XIX	X/D
B. asacch	1557[a]	1337	1614	1042	1470	1404.4/227.7
XIX_9 (B. intermedius)	566	385	1804	140	1582	895.4/747.8
B. intermedius	996	937	1555	722	1431	1128.2/351.0
B. mel CS1	1028	822	1614	1243	1580	1257.4/344.1
B. frag 382	1060	709	1200	432	819	844.0/300.8
B. frag	572	166	584	67	511	380.0/244/6
B. ovatus	462	315	740	109	968	518.8/340.4
Mean =	891.6	667.3	1301.6	536.4	1194.4	
S.E. =	336.4	251.8	490.9	202.4	450.5	

[a] optical density at 490 nm - average of duplicates

Table 31

ELISA Measuring IgG

Bacteroides strain	XII	Control	XIV	Control	XV	Control	XVI	Control	XIX	Control	ANUG X/SD	Control X/SD
B. asacch.	772	895	1320	1386	1545	1095	1179	684	1407	831	1244.6/295.8	978.2/271.6
XIX9 (B. intermedius)	899	1247	1296	972	1705	528	775	708	1654	1030	1265.8/424.2	897.0/281.8
B. intermedius	1538	1824	1643	1670	1744	1750	1574	1582	1677	1637	1635.2/81.9	1692.6/95.4
B. mel CS1	1609	1871	1506	1790	1683	1677	1763	1920	1736	1567	1659.4/104.0	1765.0/143.9
B. frag 382	892	606	673	1263	1470	1189	495	674	1050	751	882.5/424.0	896.6/306.2
B. frag	813	261	--[b]	700	499	385	332	271	858	398	500.4/355.1	403.0/177.6
B. ovatus	200	62	1630	--	532	751	883	529	1518	309	952.6/617.8	330.2/315.4
Mean	960.4	966.6	1152.6	1111.6	1311.1	1053.6	1000.1	909.7	1414.3		931.9	
S.E.	362.4	364.7	434.9	419.4	494.8	397.4	377.4	343.3	533.7		351.6	

[a] optical density at 490 nm
[b] no activity

Studies on Spirochetes from ANUG Lesions

Attempts were made to determine the number of rods, cocci and spirochetes in the plaque samples and to cultivate spirochetes from these samples. Plaque debris and extracted teeth were studied by transmission and scanning electron microscopy, respectively, to evaluate the spirochetes involved. In order to characterize the spirochete isolates serological studies; sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); and enzyme-linked immunoelectrotransfer blot (EITB) analyses were performed. In addition to these studies, investigations of the surface antigens were undertaken. They include the isolation of the outer sheath, determination of the proteins within and determining if the oral anaerobic spirochetes adsorb host proteins. The materials and methods and results of these studies follows.

MATERIALS AND METHODS

Dark-field microscopic counts

The RTF plaque samples used in the culture attempts were vortexed for 30 sec using a vortex-genie and then a drop of each plaque suspension was placed in the center of a Petroff-Hausser Counting Chamber which had been covered with a No. 1.5 cover glass. The counting chamber was then allowed to stand 15 min at room temperature and was subsequently examined under dark-field at a magnification of 500 X using a Leitz Dialux 20 microscope. All bacteria observed in 80 squares of the counting chamber were counted and divided into 3 categories: (1) rods, (2) cocci, and (3) spirochetes respectively. All counts were completed within 24 hours of plaque collection.

Videotaping of ANUG plaque specimens

Plaque samples, taken by curretage, were applied directly to a clean glass microscope slide. Each specimen was then covered with a no. 1.5 cover glass, sealed with petrolatum, and viewed under darkfield and phase contrast optics using a Leitz Dialux 20 microscope. Images of these specimens were recorded using a Hitachi camera (model HV177U) and a Sony U-matic video recorder.

Isolation of Spirochete Strains

Subgingival plaque samples were collected by a periodontist from clinic patients. These samples were placed in a tube containing prereduced Medium A Broth (described below) and transported to a Coy anaerobic chamber. This chamber was maintained at a temperature of 35° C and with an atmosphere of 85% nitrogen, 10% hydrogen and 5% carbon dioxide. The samples were treated as described by Leschine and Canale-Parola (122) with the plaque sample serially ten-fold diluted in 13 x 100 screw-capped tubes containing the NOS medium with 1-2 ug/ml of rifampin and 0.3% agar. The tubes were then incubated inside the chamber for 7-14 days at which time the resulting individual isolated spirochetal colonies were picked and examined by dark-field microscopy to determine the presence of spirochetes. Positive colonies were streaked on the appropriate medium in the chamber to obtain isolated clones or rediluted as before in fresh NOS media. A portion of the sample was also placed on 25 mm-0.45 um pore size Millipore filters on the surface of Medium A containing 1.0% agar. The spirochetes migrated through the filters and the larger contaminating bacteria remained on the filter surface. Spirochetes developed as a veil of growth and the leading edge of the growth was removed by taking a plug of agar with a Pasteur pipette and restreaking on solid medium within the anaerobic chamber to obtain isolated clones (Fig. 1).

Spirochete Cultivation

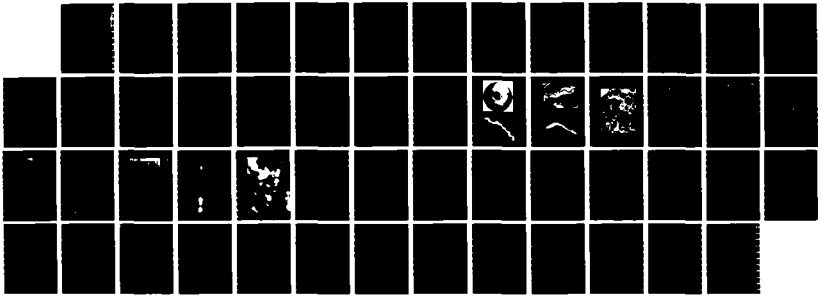
The oral spirochete strains which we have require slightly different growth media. The composition of the media used follows:

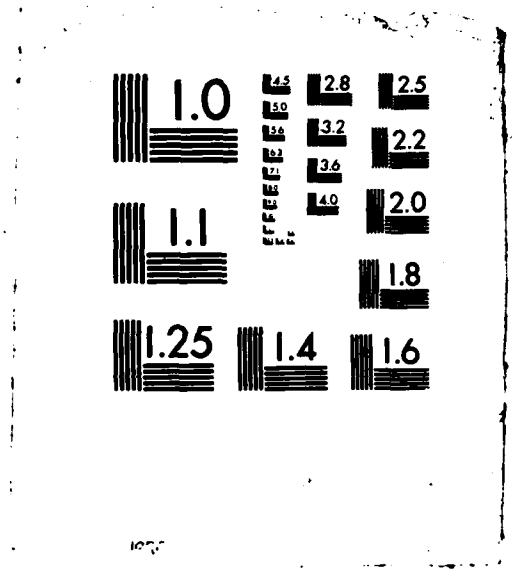
Medium A

Component	Amount
Mycoplasma Broth (BBL)	21 g/l
Thiamine Pyrophosphate	10 ug/ml
Sterile Rabbit Serum	
Agar (Oxoid)	10.0 g/l

AD-A172 119 ACUTE NECROTIZING ULCERATIVE GINGIVITIS: MICROBIAL AND IMMUNOLOGIC STUDIES(U) MARYLAND UNIV BALTIMORE DENTAL SCHOOL W A FALKLER 05 AUG 84 DAMD17-80-C-0101 2/2

UNCLASSIFIED F/G 6/5 NL




1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6

Heat inactivated (56° C for 30 min) 10% V/V

pH 7.4

NOS Medium

NOS-Part A

Component	Amount
Heart infusion broth (Difco)	1.25 g
Trypticase (BBL)	1.0 g
Yeast Extract (Difco)	0.25 g
Sodium thioglycollate	0.05 g
L-cysteine-HCl	0.1 g
L-asparagine	0.025 g
Agar (Oxoid)	0.3 g
Distilled water	90 ml

Final pH 7.0

NOS-Part B

Component	Amount
2% $NaHCO_3$ (rifampin for isolation 20 ug/ml) filtered sterilized.	1.0 ml/9.0 ml Part A

VFA-TPP-Serum

1.5 ml 0.2% thiamine pyrophosphate

1.0 ml VFA soln

10 ml sterile, heat inactivated rabbit serum. 0.25 ml/9.0 ml Part A

Volatile Fatty Acid Solution (VFA)

500 ul Valeric Acid

500 ul Isovaleric Acid

500 ul Isobutyric Acid

500 ul DL-2-Methyl Butyric Acid

T. vincentii medium

I. Add to 88 mls of DH_2O (g/88 mls):

Heart infusion broth (Difco)	0.5
Trypticase (BBL)	1.0
Yeast Extract (Difco)	1.0
Gelatin	1.0
$(NH_4)_2 SO_4$	0.05
$Mg SO_4$	0.01
$K_2H PO_4$	0.12
$KH_2 PO_4$	0.10
NaCl	0.10
Glucose	0.1 g
L-Cysteine - HCl	0.1 g

II. Adjust to pH 7.4 w/ 10 N KOH

III. Autoclave

IV. Add (0.33% TPP stock) 1 ml

Stock 0.33g, TPP/100 DH_2O

Filtered sterilized

V. Add normal rabbit serum 10 ml

VI. Heat inactivate 30 min at 56° C

Scanning electron microscopy (SEM) of extracted teeth

Freshly extracted teeth were immediately immersed in 0.2 M sodium cacodylate buffer, pH 7.4, containing 2% glutaraldehyde (GA) and fixed at 4° C for 16 to 24 h. Following the prefixation in GA, the specimens were washed 3 times with 0.2 M cacodylate buffer and transferred into 0.2 M sodium cacodylate with 1.0% osmium

tetroxide (pH 7.2) for 2 h at room temperature and then washed 3 times with buffer. The specimens were then dehydrated in a graded series of ethyl alcohol (30%, 50%, 70%, 90%) for 15 min exchanges, and then into 100% ethanol for two fifteen min exchanges. The specimens, in 100% ethanol, were then transferred to a Tousimis 810 critical point dryer and critically point dried with liquid CO_2. The dried tissue was mounted on specimen stubs with a silver conductive paint and sputter coated with gold-palladium with a Technics Hummer VI sputter coater and examined with a JEOL T-200 SEM. Final images were recorded with Polaroid Typd 55 P/N film.

Transmission electron microscopy (TEM)

Plaque debris obtained from each patient was dispersed in 1 ml of sodium phosphotungstate, pH 7.3. A drop of the suspension was placed onto a formvar-carbon coated 300 mesh copper grid and examined in a Siemens IA electron microscope at 80 kV. Electron micrographs were recorded on Kodak electron image plates.

Each sample examined in the TEM was evaluated for the types of spirochetes present based on the number of axial filaments originating from each end of the cell. In each case, a minimum of 20 spirochetes was counted per sample and the number of filaments and their arrangement recorded. During this analysis selected electron micrographs were taken to record the types of spirochetes found in the samples.

Preparation of Antisera

Specific immune rabbit sera was prepared in New Zealand White rabbits for some of the spirochete strains listed in Table 32 as well as other isolates. The protocol used for preparing antiserum for these strains consists of weekly intravenous inoculations of 2.5 ml of a 6 to 7 day old broth culture for 4 weeks. Five days after the last immunization, blood was collected by cardiac puncture on three successive days and the serum separated and the three bleedings pooled. Microscopic agglutination tests (MA) were done to determine the titer of the pooled antiserum and to determine commonality of surface antigenic components. In the past we have obtained MA titers of 1:10,000 or

greater using this immunization schedule.

Serological Relationships Among Spirochete Isolates

All of the spirochete isolates were grown in the appropriate liquid medium and the cell density adjusted to approximately 1 x 10^8 cells/ml. Microscopic agglutination analysis was then performed by the method described by Gochenour et al. (123). Briefly stated, the MA test was performed in the following manner. Serial ten fold dilutions (0.5 ml volume) ranging from 1.5 to 1:500,000 of each rabbit antiserum were prepared using 0.85% NaCl as the diluent. This was followed by the addition of 0.5 ml of an actively growing 5-7 day broth culture of either the homologous or heterologous spirochetal strains. The tubes were kept at 25° C for 2 hours with intermittent agitation every 15 min. Controls included non-immune rabbit serum reacted against each isolate as well as saline, instead of antiserum to test for autoagglutination. Following the 2 hour incubation period, one drop of each reaction mixture was placed on a microscope slide and agglutination patterns were recorded at 100 diameters with a Leitz Dialux 20 microscope equipped with a low power dark-field condenser. The reciprocal of the highest dilution of each serum which resulted in agglutination of at least 50% of the spirochetal cells was recorded as the end point.

Sodium Dodecyl sulfate-Polyacrylamide Gel Electrophoretic Analysis of Spirochetal Antigens (SDS-PAGE)

The SDS-PAGE analysis of our isolates was performed in the following manner. The gels and solutions used were modifications of those previously described by Swindlehurst, et al. (124), and Payne (125). The spirochete isolates were grown in 175 ml broth cultures for 6-7 days, harvested by centrifugation at 10,000 x g for 20 min. The pellets were washed three times with 0.1M tris buffer pH 6.8 and were suspended in the same buffer to an optical density of 1.5 at 650 nm. The cells were then frozen and thawed 4 times, divided into 50 ul fractions and either stored at -20° C or used immediately. Samples were then digested by boiling 25 ul of each cell suspension in a

mixture containing 0.6 M tris, 2% SDS, 5% mercaptoethanol, 10% sucrose, and 0.001% bromophenol blue, for 2 minutes. After cooling to room temperature, twenty microliters of each suspension were added to wells of a 1.5 mm vertical discontinuous polyacrylamide gel slab composed of a 5% acrylamide stacking gel (0.125 m Tris HCl, pH 6.8) and a 12.5% acrylamide separating gel (0.375 M Tris HCl, pH 8.8). Electrophoresis was carried out using a BRL vertical gel electrophoresis system (model V16) at 150 volts constant voltage, in a Tris-glycine buffer (0.025 M Tris - 0.192 M glycine, pH 8.3) until the bromophenol blue tracking dye approached the bottom of the gel.

The gel was removed from its glass plate sandwich and fixed in an aqueous solution containing 10% trichloroacetic acid, 5% sulfosalicylic acid (W, W, V) for 1 h. The fixative was then removed and the gel allowed to equilibrate in an aqueous solution of 25% methanol, 5% acetic acid (V, V, V) for 30 min. Following equilibration, the gel was then stained for 6 h in 0.1% Coomassie Brilliant R-250 Blue in an aqueous solution of 25% methanol and 5% acetic acid and then destained in an aqueous solution of 25% methanol, 5% acetic acid until the background was clear. Once the gels were destained, they were photographed with Polaroid 55 P/N film to record the gel patterns.

Enzyme-Linked Immunoelectro-Transfer Blot (EITB)

Following electrophoresis, the SDS-gel was placed on nitrocellulose paper (0.45 um pore size, Millipore, Bedford, Ma) and the two were inserted between 6 sheets of 3 mm filter paper, 2 scotch-brite pads, 2 Lucite electrode supports and placed in a electrophoretic chamber with the nitrocellulose paper facing the anode. The protein transfer chamber buffer consisted of 0.025M Tris, 0.193 M glycine, 20% methanol at pH 8.35. A voltage gradient of 10 V/cm was applied for 13 h at 4^{o} C. Specific spirochetal antigens were detected as follows: after electrophoretic transfer, the nitrocellulose sheet was removed and was washed with 0.01 M Na_2HPO_4/NaH_2PO_4, 0.15M NaCl-0.3% Tween 20 pH 7.2 (PBS-Tween). The sheet was then incubated with antiserum at a dilution of 1:50 in the PBS-Tween buffer at room temperature with constant rocking.

The sheet was washed again with PBS-Tween at room temperature and incubated for 1 hour with a 1:1000 dilution of horseradish peroxidase labeled goat anti-rabbit immunoglobulins (IgA, IgG, IgM, H & L chains). The nitrocellulose sheet was again washed as before. To detect the antigen-antibody reactions, 4-chloro-1-Naphtol-H_2O_2 was used as indicator and substrate. The indicator/substrate reagent was prepared by adding 20 mls of a 60 mg 4-chloro-1-naphtol/20 ml methanol stock solution and 60 ul of 30% H_2O_2 solution to 100 mls of the PBS-Tween buffer and allowing it to react with the nitrocellulose sheet for 30 min. The reaction was then stopped with distilled water. The EITB nitrocellulose sheet was photographed wet, using Polaroid 4 x 5 in, type 55 positive-negative film.

Outer Sheath Isolation

The procedure used to isolate the outer sheath of the oral spirochetes was a modificatioon of the technique described by Masuda and Kawata (126). Briefly, the procedure involved harvesting cells from a 5-7 day 1 liter broth culture. Washing the cells three times with 50 mM Tris-hydrochloride buffer (pH 7.2), then suspending the resulting cell pellet in 60 ml of the same buffer containing 2 mM $MgCl_2$ and DNAse I at a concentration of 2 ug/ml. The cells were then disrupted by 20 cycles of freezing in dry ice-acetone and subsequently thawing in 37° C water. The disrupted cell suspension was centrifuged (8,000 xg, 10 min) to remove unbroken cells and large cell debris. The supernatant was collected and centrifuged at 25,000 x g for 30 min to obtain a crude preparation of the outer sheath. After washing the crude outer sheath preparation with Tris-hydrochloride buffer (3 times), the crude preparation was suspended in 60 ml of 0.1 M sodium acetate-hydrochloride buffer (pH 3.0) to dissociate contaminating periplasmic flagellar proteins. The outer sheath protein suspension was then agitated gently on a magnetic stirrer for 2 h, and then centrifuged at 25,000 for 30 min. The resulting pellet was suspended in 6 ml volume of Tris-hydrochloride buffer stirred overnight at 40°, and then layered on top of a 35 to 50% linear sucrose density gradient made in the same buffer, and centrifuged at 195,000 x g for 2 hours in a SW 50.1 rotor. After the

centrifugation the resulting outer sheath band was pulled off by puncturing the side of the tube with a syringe, diluted with Tris-hydrochloride buffer, and centrifuged at 25,000 x g for 30 min. The resulting pellet was then stained for EM and prepared for SDS-PAGE.

Results

Microscopic Analysis

Dark-field microscopic analysis of ANUG plaque samples revealed an abundance of rods, cocci, and spirochetes. Counts of the numbers of each morphological type in the plaque samples with the Petroff-Hausser counting chamber resulted in the percent composition of each type as shown in Table 32 for each of the 17 samples evaluated. Permanent video tape records of the morphological types seen under dark-field and phase contrast microscopy were also prepared using television photomicrography for selected patient samples.

The distribution of types of spirochetes bases on periplasmic flagella numbers and arrangement by negative staining TEM for selected ANUG samples is shown in Table 33. The predominate spirochetes appear to be the type with the "2-4-2" axial filament arrangement which are presumable Treponema denticola. The next most abundant spirochetes observed were equally divided among the large size spirochete of the "12-24-12", "6-12-6", and "8-16-8" classes. Examples of some of these types is shown in Figures 2-4.

Videotapes displaying ANUG plaque specimens using dark-field and phase contrast optics are available upon request. Overall, a consistent microbial flora was observed consisting of highly motile rods, fusiform shaped bacteria and several types of spirochetes. Polymorphonuclear leukocyctes (PMN) were also observed.

Scanning electron microscopic analysis was performed on freshly extracted teeth from one patient who was diagnosed as having juvenile periodontitis with super imposed

Table 32

Percent Morphological Composition of ANUG Plaque

Sample Number	% Rods	% Cocci	% Spirochetes	Total Organisms
1	28.60	21.31	41.80	122
2	52.90	23.22	23.87	155
3	48.64	30.63	20.72	111
4	48.14	31.85	20.00	270
5	48.75	23.75	27.50	160
6	38.91	24.63	36.45	609
7	43.94	18.94	37.12	528
8	50.34	49.65	0.00	145
9	35.48	17.35	47.17	513
10	39.55	32.88	27.61	268
11	51.38	19.89	28.73	181
12	37.19	38.11	24.69	328
13	42.86	50.00	7.14	266
14	42.80	21.02	37.31	528
15	38.80	26.86	34.33	335
16	46.39	38.14	15.46	97
17	32.01	25.30	42.68	328
Avg. %	42.75%	29.02%	29.97%	4994

Table 33

Distribution of Spirochetes in ANUG Debris

Axial Filament Arrangement

Patient #	1-2-1	2-4-2	3-6-3	4-8-4	6-12-6	8-16-8	12-24-12	16-32-16
1	4	8	0	1	8	6	9	0
2	2	11	0	0	3	0	6	0
3	0	0	2	3	5	1	7	5
4	0	5	0	0	1	7	11	2
5	2	8	1	2	0	9	2	0
6	2	16	0	2	2	10	4	1
7	7	16	0	3	16	0	8	0
8	10	15	2	1	12	0	10	0
9	3	13	1	3	10	19	1	0
Totals	30	84	6	15	57	52	58	8
% of Total	9.7	27.0	2.0	4.8	18.4	16.8	18.7	2.6

ANUG. Several teeth were extracted and the adherent plaque evaluated by SEM. Figure 5 is an SEM of tooth #15 showing that the predominate flora found was that of spirochetes.

Isolation and Cultivation of Spirochetes from ANUG Lesions

Significant progress has been made in isolating and cloning new spirochete strains from ANUG lesions. We have been successful in isolating 7 new strains (Table 34), all of which have been found to be T. denticola. These ANUG isolates were compared to other strains of spirochetes which we have isolated from patients with other periodontal problems or have been acquired from other investigators. Table 35 is a list of the known isolates and their periplasmic flagella arrangement, currently available in our laboratory. Figure 1 represents the veil like growths observed with our ANUG isolates on PPLO-serum medium.

Serological analysis of spirochetes from ANUG and other spirochetes. Microscopic Agglutination (MA).

The MA test was used to determine whether the various ANUG isolates shared common surface antigens among themselves as well as among other T. denticola, T. pectinovorum and T. vincentii isolates. As can be seen in Table 36, MA titers ranging from 10^3 to 10^6 were obtained when each isolate was reacted against its homologous rabbit antiserum. When each isolate was reacted against the heterologous antigens, several consistent patterns were found: (a) there was a variety of cross-reactivity displayed within each of the 3 species of spirochetes with a range of no reactivity (0) to a titer as high as 10^5 and (b) there was no serological cross-reactivity or sharing of surface antigens among T. pectinovorum, T. denticola and T. vincentii. Non-immune rabbit serum did not agglutinate any of the isolates. Thus, microscopic agglutination analysis of homologous and heterologous T. denticola strains suggest that some strains of T. denticola share cross-reactive surface antigens, while others do not. While microscopic agglutination analysis of homologous and heterologous T. pectinovorum strains suggest that these strains share a high degree of cross-reactive surface antigens and microscopic agglutination analysis indicated that there is no serological cross-reactivity or sharing of surface antigens among T. denticola, T. pectinovorum and T. vincentii strains.

Table 34

Oral Spirochetes Isolated from ANUG Patients

Organism	Strain Designation	Periplasmic Flagella Arrangement	Source
T. denticola	MS	2-4-2	ANUG
T. denticola	DW	2-4-2	ANUG
T. denticola	MI	2-4-2	ANUG
T. denticola	22	2-4-2	ANUG
T. denticola	23	2-4-2	ANUG
T. denticola	1022	2-4-2	ANUG
T. denticola	Rose	2-4-2	ANUG

Table 35

Spirochete Strains Available in our Laboratory for Comparative Studies

Organism (a)	Strain Designation (b)	Periplasmic flagella arrangment	Source (c)
T. denticola	T	2-4-2	AP
T. denticola	TT	2-4-2	UMDS* AP
T. denticola	W	2-4-2	UMDS AP
T. denticola	11	2-4-2	UMDS AP
T. denticola	JZ	2-4-2	UMDS AP
T. denticola	MS	2-4-2	UMDS ANUG
T. denticola	DW	2-4-2	UMDS ANUG
T. denticola	MI	2-4-2	UMDS ANUG
T. denticola	JP428	2-4-2	UMDS GJP
T. denticola	USA	2-4-2	AP (Rhesus monkey)
T. denticola	23	2-4-2	UMDS ANUG
T. denticola	22	2-4-2	UMDS ANUG
T. denticola	Rose	2-4-2	UMDS ANUG
T. denticola	1022	2-4-2	UMDS ANUG
T. denticola	JD_1	2-4-2	WLC
T. denticola	JD_2	2-4-2	WLC
T. denticola	JD_3	2-4-2	WLC
T. denticola	8A	2-4-2	WLC
T. denticola	2513	2-4-2	U. PA
T. denticola	2516	2-4-2	U. PA
T. denticola	2519	2-4-2	U. PA
T. denticola	CD1	2-4-2	U. PA
T. denticola	MRB	2-4-2	CDC
T. denticola	USA	2-4-2	Monkey
T. vincentii	N-9	5-10-5	JOHNS HOPKINS U.
T. vincentii	LA1	5-10-5	U. PA
Unnamed Treponema sp	10A	1-2-1	WLC
T. pectinovorum	P2	1-2-1	U. MASS
T. pectinovorum	P3	1-2-1	U. MASS
T. pectinovorum	P4	1-2-1	U. MASS
T. pectinovorum	P5	1-2-1	U. MASS
T. pectinovorum	P8	1-2-1	U. MASS

Table 35 (con't.)

a. The organisms labelled T. denticola were identified as such based on periplasmic flagella arrangement, dark-field morphology and GLC analysis of metabolic end products.

The organisms labelled T. pectinovorum were identified as such based on periplasmic flagella arrangement, dark-field morphology and nutritional requirements.

The organisms labelled T. vincentii were identified as such based on periplasmic flagella arrangement, dark-field morphology and nutritional requirements.

b. T. denticola strains W and 11 are now ATCC #33520 and #33521 respectfully.

c. T. denticola, USA was isolated from a Rhesus monkey with periodontal disease.

T. vincentii N-9 was obtained from Dr. Paul Hardy, Johns Hopkins U.

T. pectinovorum strains P2, P3, P4, P5 and P8 were obtained from Dr. E. Canale-Parola, U. Mass., who had isolated them from the oral cavity of laboratory workers. The medium used had pectin as the sole source of carbon.

T. vincentii strain LA1 and T. denticola strains 2513, 2516, 2519 and CO1 were obtained from Dr. Max Listgarten, U. of Pa.

T. denticola strains JD, JD2, JD3, 8A and strain 10A were obtained from Dr. Ron Mink, Warner Lambert Co., N.J.

T. denticola strain MRB was obtained from Ms. Elizabeth Hunter, CDC, Atlanta.

UMDS - University of Maryland Dental School

Table 36. Microscopic Agglutination Titers[a] of Rabbit Antisera Against the Homologous and Heterologous Strains of Oral Spirochetes

	T. denticola								*T. pectinovorum*			*T. vincentii*
Antisera / Antigen	MS	JP	USA	TT	11	W	22	MRB	P2	P3	P8	N-9
MS	10^6	10^2	0	10^2	0	10^2	10^1	0	0	0	0	0
JP	0	10^4	0	10^1	0	10^1	10^2	--	0	0	0	0
USA	10^2	10^2	10^4	10^2	10^1	10^5	10^3	10^2	0	0	0	0
TT	10^2	10^1	0	10^3	10^1	10^1	0	10^2	0	0	0	0
11	10^2	10^1	0	10^1	10^4	10^1	0	10^2	0	0	0	0
W	10^3	10^3	10^3	10^1	10^1	10^6	10^2	10^1	0	0	0	0
22	10^2	10^2	10^2	10^2	0	10^3	10^3	0	0	0	0	0
MRB	10^3	0	0	0	0	0	10^2	10^3	0	0	0	0
DW	10^3	10^1	0	0	10^4	0	10^1	10^3	0	0	0	0
JD_1	0	0	10^2	0	0	0	0	0	--	--	--	0
8A	0	0	10^2	0	0	10^3	0	0	--	--	--	0
P2	0	0	0	0	0	0	0	0	10^4	10^3	10^3	0
P3	0	0	0	0	0	0	0	0	10^2	10^3	10^3	0
P8	0	0	0	0	0	0	0	0	10^3	10^3	10^3	0
P4	0	0	0	0	0	0	0	0	10^3	10^3	10^3	0
P5	0	0	0	0	0	0	0	0	10^3	10^3	10^3	0
N-9	0	0	0	0	0	0	0	0	0	0	0	10^3

-- Not tested

[a]/ Reciprocal of highest dilution resulting in agglutination of 50% of cells

Though the ANUG isolates tested by MA showed varying degrees of cross reactivity among themselves, this was consistent with what was observed with the rest of our T. denticola isolates.

SDS-PAGE analysis of ANUG isolates

SDS-PAGE analysis has been performed on some of the ANUG spirochetal isolates. SDS-PAGE patterns obtained for each isolate has been compared with those of known spirochetal strains. Figure (6) and (7) are examples of typical gel patterns obtained using this technique. A comparison of the major and minor proteins can be made and those organisms that are identical display identical gel patterns. Fig (6) Lanes 1-4 are known T. denticola isolates; lanes 5-7 are ANUG isolate MS, Rose and 22; lanes 8-12 represent strains of T. pectinovorum; and 13 contains T. vincentii, N-9. As one can see strains that are identical will display identical gel patterns i.e. lanes 1 and 2 and lanes 11 and 12 whereas strains of the same species can display significant differences in protein profiles (lanes 2 and 3). The ANUG isolates chosen for this particular experiment (lanes 5-7) have distinctly different gel patterns and therefore probably represent three different strains. Fig (7) displays gel profiles of various strains of T. denticola. Lanes marked 4, 6, 7 and 8 are ANUG isolates which show consistent protein profiles seen in the other T. denticola isolates.

Enzyme-linked Immunoelectro-Transfer Blot (EITB)

The EITB technique was used to determine whether the various strains of spirochetes used in this study shared any protein antigens. As can be seen from SDS-PAGE analysis (Figure 6 and Figure 7), there are approximately 25 to 35 proteins in the 3 species which share migrational similarities in a 12.5% PAGE separating gel.

When sera produced against T. denticola strains W and MS were reacted with nitrocellulose sheets containing the antigens of all 3 species, a variety of reactions was observed (Fig. 8 and Fig. 9). Twenty-seven to thirty antigens were identifiable in the homologous reaction. The heterologous reaction of T. denticola sera and T.

pectinovorum antigens displayed 14 to 17 cross-reactive antigens. While the heterologous reaction of T. denticola sera and T. vincentii antigens revealed 12 to 14 immunologic cross-reactive antigens.

The heterologous reaction of T. denticola sera and other T. denticola strains showed a wide variety of cross reactivity among their antigens indicating various strain antigenic differences.

Conversely when homologous T. pectinovorum sera was used (Fig. 10) approximately 34 antigens were identified. While with the heterologous reaction of T. pectinovorum sera with T. denticola and T. vincentii antigens 14 to 19 and 15 to 17 cross-reacting antigens were seen respectively.

The heterologous reaction of T. pectinovorum and other T. pectinovorum strains showed little variation in antigen cross-reactivity, thus suggesting their serologic similarity.

When the homologous T. vincentii reaction was carried out 30 to 32 proteins were observed (Fig. 11). The heterologous reaction of T. vincentii sera with T. denticola and T. pectinovorum antigens revealed 15 to 18 antigen and 13 to 15 antigens sharing antigenic cross-reactivity respectively.

Adsorption of Rabbit Serum Proteins

To determine if our spirochete isolates adsorbed serum proteins from the spirochete medium, goat anti-rabbit albumin conjugated with horseradish peroxidase was used as the primary antibody in EITB analysis. Figure 12 shows the result when such an experiment was performed. Lanes 1-8 are T. denticola isolates W, 11, MS, JP, 2513, 1022, USA and JD_3; Lanes 9 and 16 are BSA (1 mg/ml) Lanes 10-13 are T. pectinovorum P8, Treponema sps. 10A, and T. vincentii LAI and N-9 respectively. Lanes 14 and 15 are rabbit serum diluted 1:20 and diluted 1:40, respectively and lastly lanes 17 and 18 contain T. denticola medium undiluted and 1:40 respectively. From this analysis many of the strains do seem to be adsorbing albumin. Those that show an albumin band are T.

denticola W, 11, JP, 2513, ANUG isolate 1022, USA and JD_3. Treponemal species WA, T. vincentii LA1 and N-d show a light albumin band when compared with the BSA, rabbit serum and medium positive controls, ANUG isolate T. denticola MS, and T. pectinovorum P8 lack an observable albumin band. T. pectinovorum P8, however, displays 3-4 lower molecular weight proteins reacting. These bands representing possible breakdown products of albumin or they could be other serum proteins recognizable by this technique. ANUG isolate 1022 shows a very strong reacting low molecular protein not seen in the other T.denticola strains. Here the same argument could be used.

The following comments can be used to summarize the EITB analysis:

(1) enzyme-linked immunoelectro-transfer blot analysis correlated well with the strain specific serologic cross-reactivity observed in each of the 3 species; (2) tThe enzyme-linked immunoelectro-transfer blot technique was able to detect cross-reactive antigens undetectable by the microscopic agglutination technique; (3) enzyme-linked immunoelectro-transfer blot analysis indicates that there are cross-reacting antigens present among these 3 species of oral anaerobic spirochete and that their location may be other than the cell surface; and (4) EITB analysis indicates that several oral spirochete species adsorb rabbit albumin.

Outer Sheath

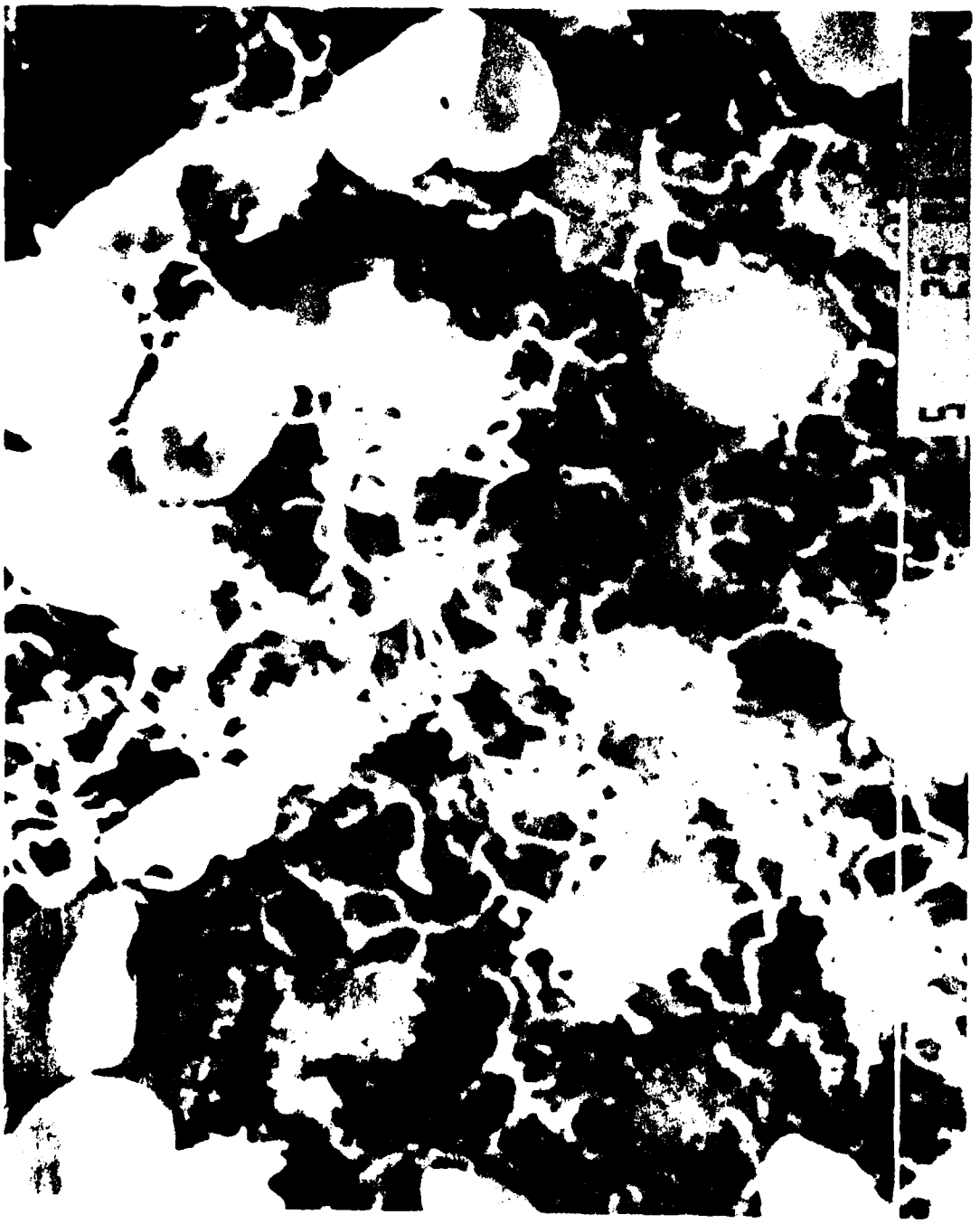
SDS-PAGE analysis of the outer sheath preparation for T. denticola strain W revealed a single major protein banding at a molecular weight position of 56 Kdal. Electron microscopy resolved large sac-like vessicles carrying a mosaic like array. There were also many smaller vessicles lacking any distinct regular array. (Fig 13).

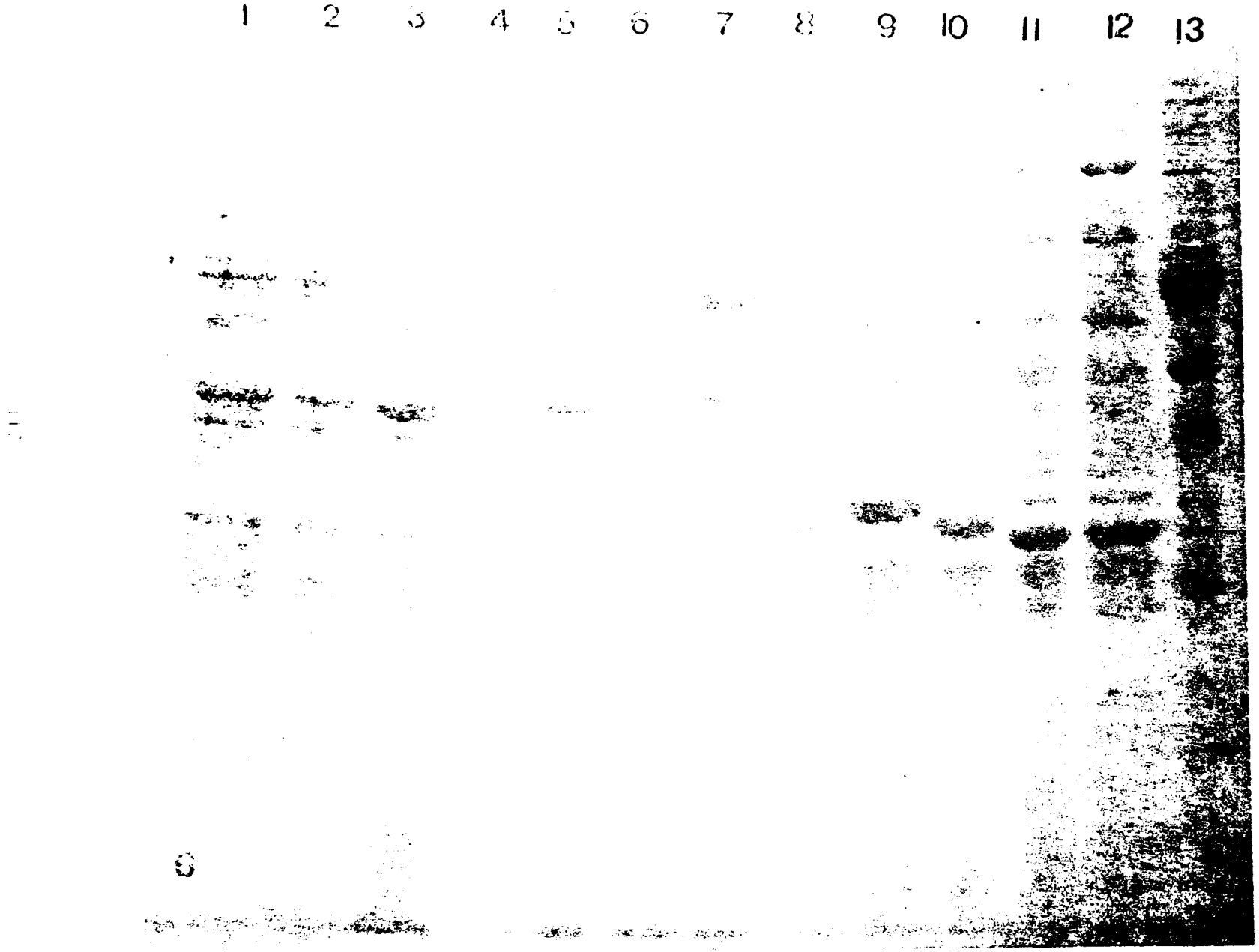
LEGENDS

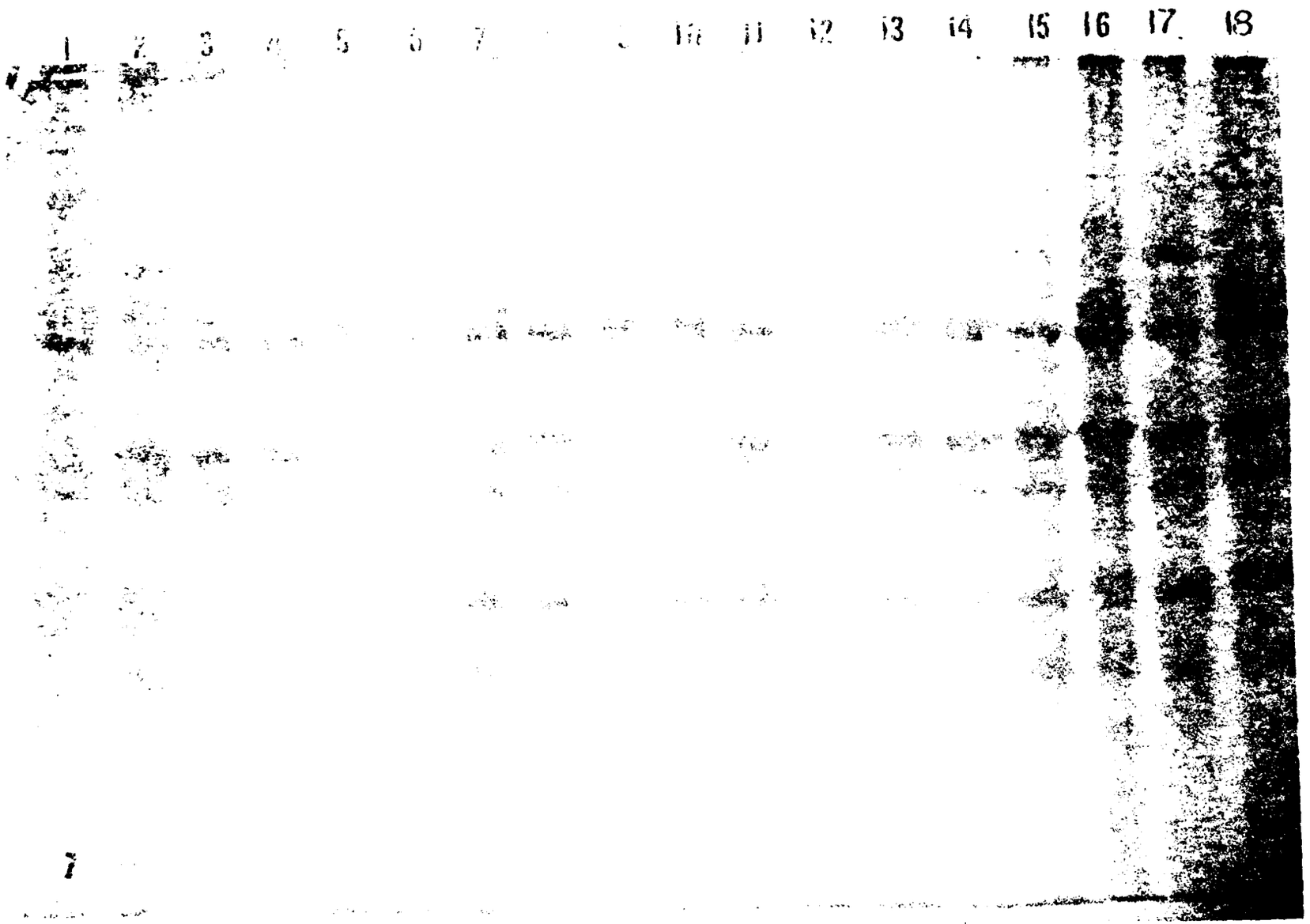
2

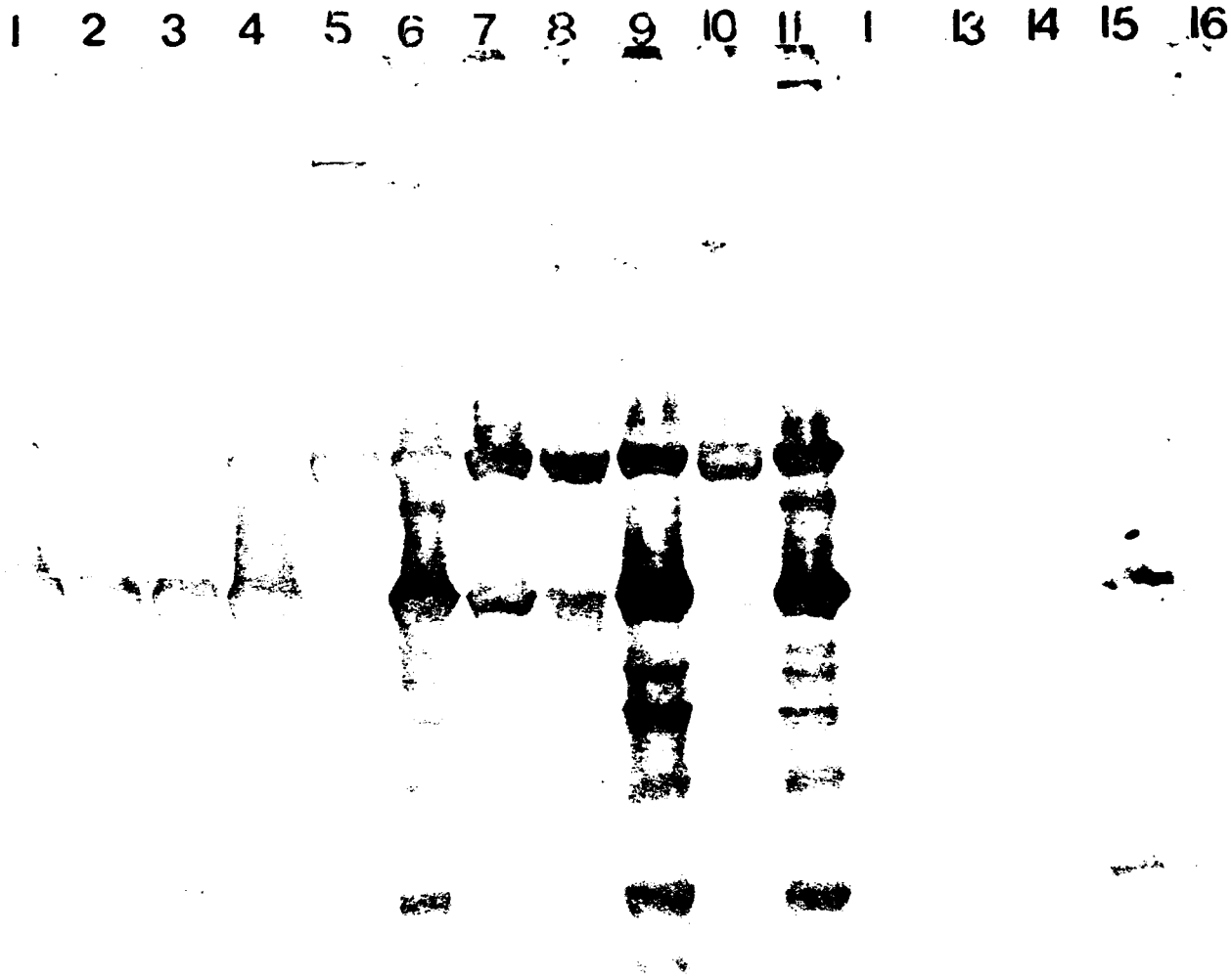
3

4

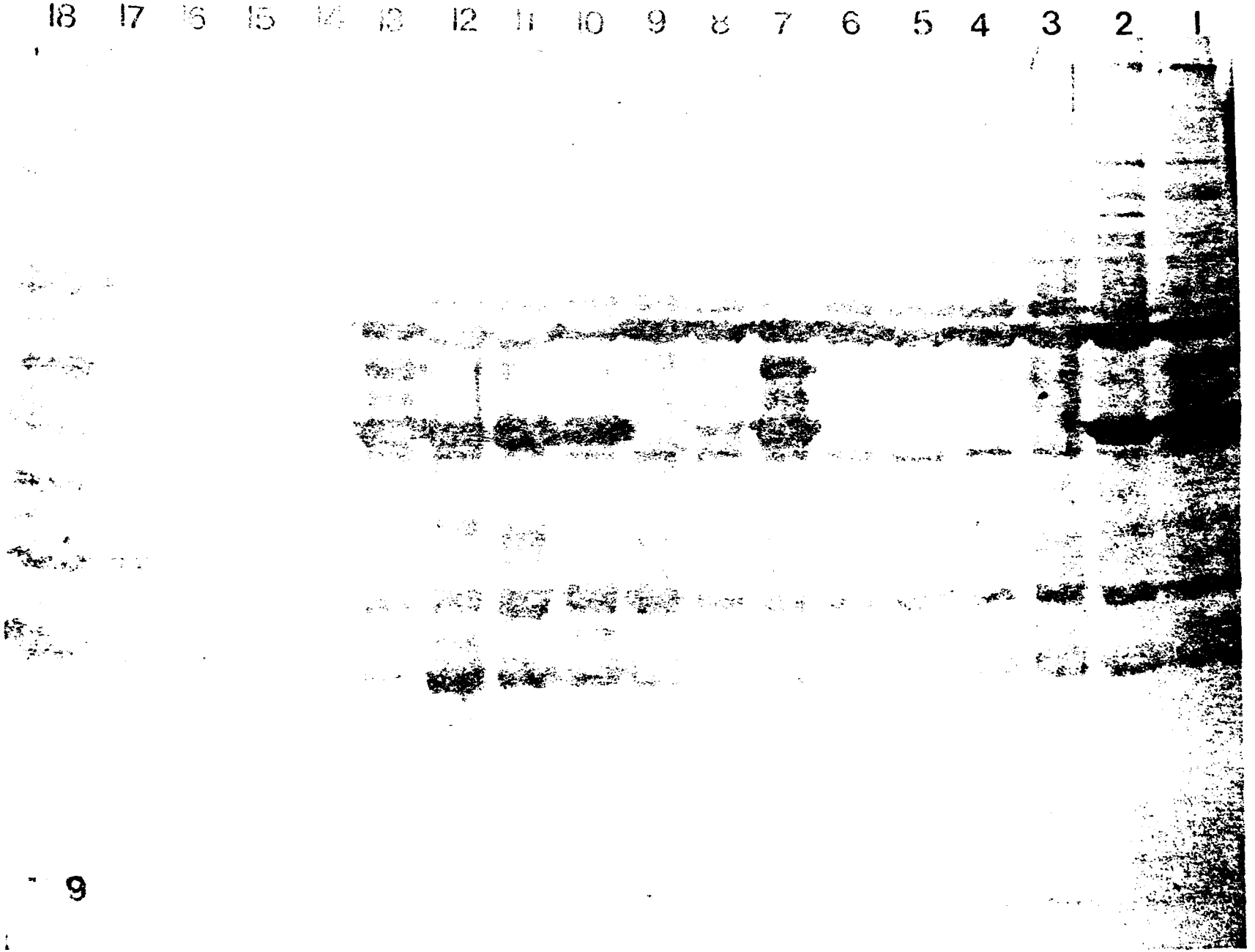


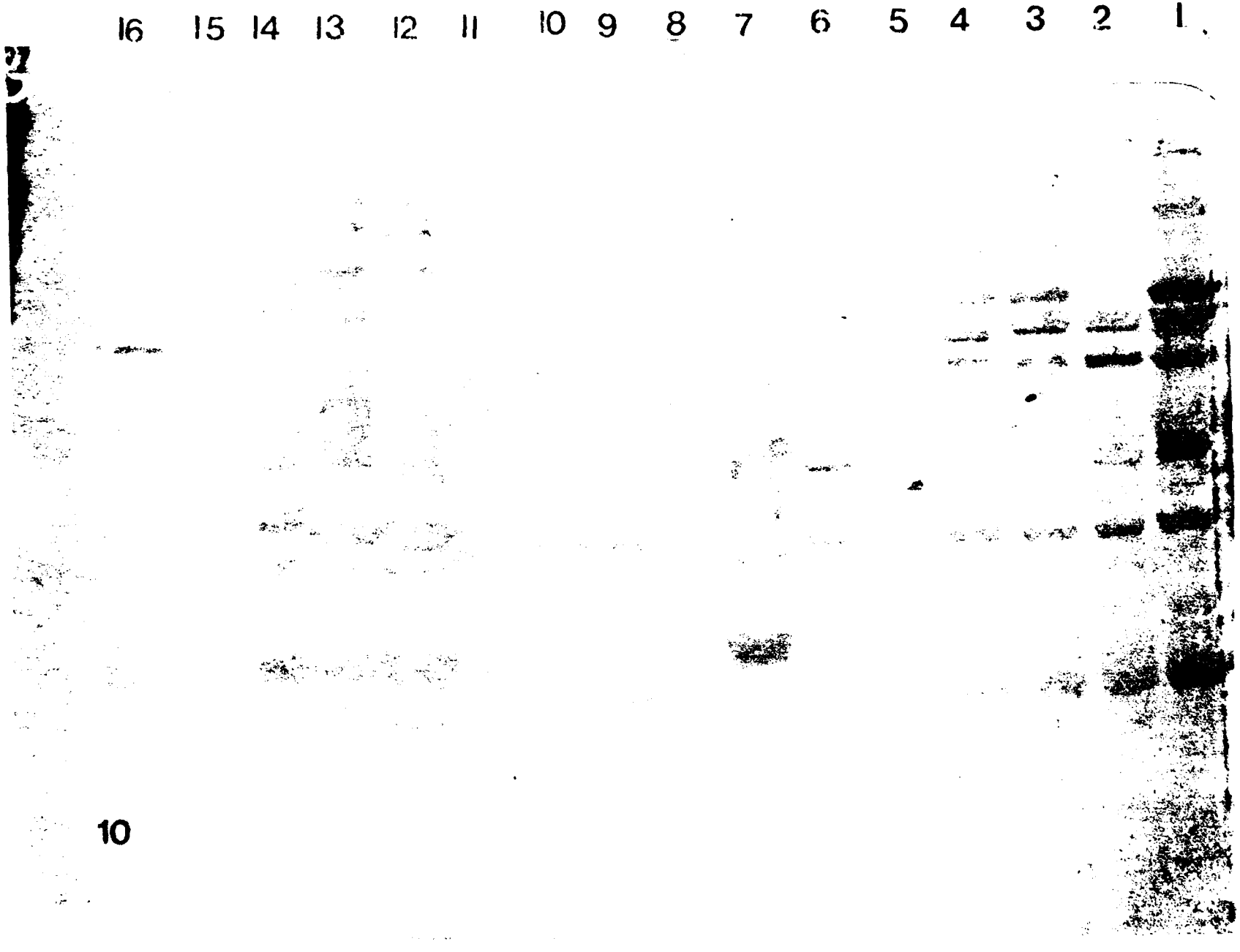

1
2
3
4
5
6
7
8
9
10
11
12
13

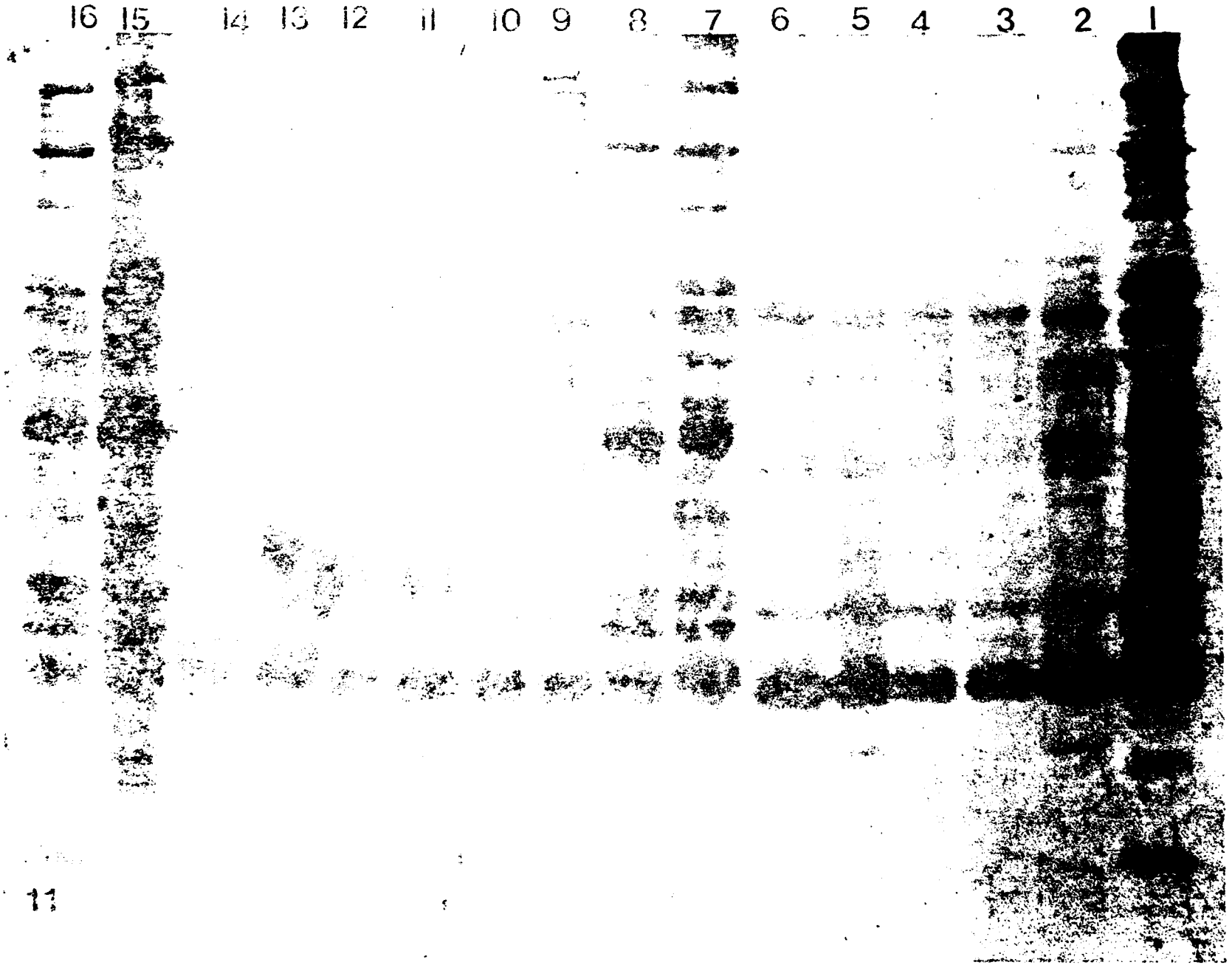

1
2
3
5
6
7
11
12
13
14
15
16
17
18

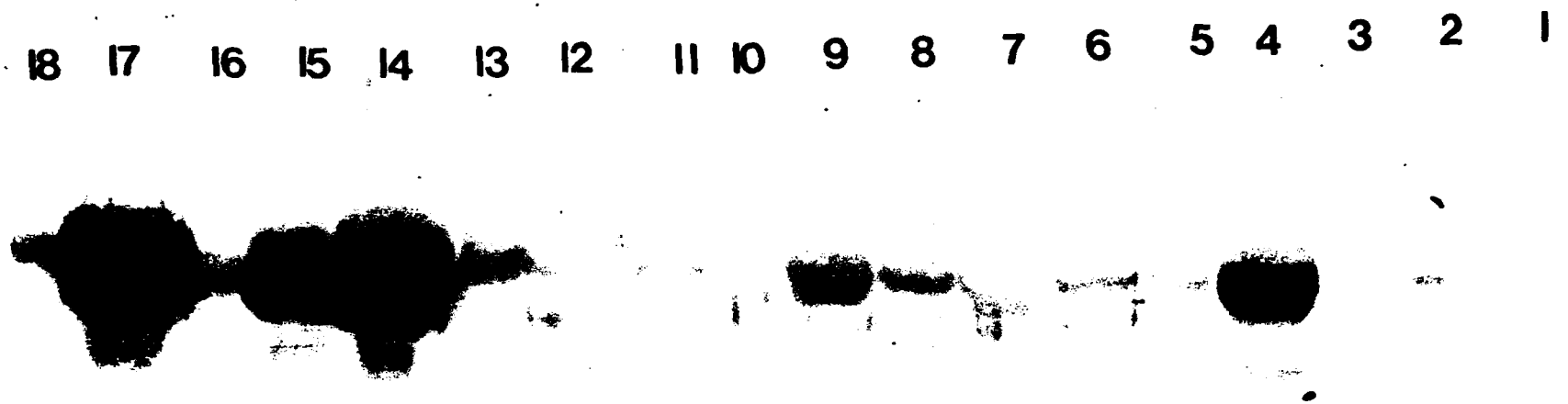


8


18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1


16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
10


16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1



12

13

Interactions of Microorganisms of Acute Necrotizing Ulcerative Gingivitis and Corticosteroids

Individuals under stress and with ANUG have been shown to have higher levels than normal of corticosteroids in their serum and urine. The microorganisms which increase in number in the ANUG lesion appear to be members of the normal oral flora. It is possible that the presence of higher levels of corticosteroids in the serum may allow increased growth and or production of pathogenic factors which may participate in the formation of the ANUG lesion.

Studies were undertaken and are currently being pursued to:

1. Determine if microorganisms associated with acute necrotizing ulcerative gingivitis (ANUG) have surface receptors for human corticosteroids. The organisms to be tested are Fusobacterium nucleatum, Treponema microdentium and Bacteroides gingivalis.
2. If receptors are observed to determine if the growth of the microorganism is increased in the presence of the steroids.

MATERIALS AND METHODS

Media

The following media were used: Crystal Violet-Erythromycin (CVE) agar; MM10 agar; and Trypticase Soy Agar (TSA) with hemin and menadione (TSAHK). CVE agar (pH 7.2) contained in g/l the following: trypticase (10.0), yeast extract (5.0), NaCl (5.0), Tryptophan (0.2), agar (15.0) and crystal violet (0.005), erythromycin (0.004), and defibrinated sheep blood (50 ml) which was added after autoclaving. MM10 contained/liter: H_2O (890 ml), 37.5 ml of 0.6% K_2HPO_4, 37.5 ml of a salt solution (NaCl, 1.2 g; $(NH_4)_2SO_4$, 1.2 g; KH_2PO_4, 0.6 g, Mg_2SO_4, 0.25 g and 100 ml H_2O), bacto-agar (15.0 g), trypticase (2.0 g), yeast extract (0.5 g), sucrose (30.0 g), KNO_3 (0.25 g). 0.05% hemin (2.0 ml) and cysteine (0.12 g), 8% Na_2CO_3 (5 ml), DL-dithiothreitol (0.1 mg) and sheep blood (20 ml) which was added after autoclaving. TSA contained 40 g/l trypticase soy agar and for the preparation of TSAHK 10 ml of 0.5 mg/ml hemin in 0.1 N NaOH, 1 ml of 0.1% menadione in 95% ETOH and 50 ml of defibrinated sheep blood was added.

Cultures and Cultural Conditions

Strains of Bacteroides gingivalis were purchased from ATCC and VPI. They were grown in brain heart infusion (BHI) broth (BBL) supplemented with hemin (5 mg/ml) and menadione (0.2 mg/ml). The Fusobacterium nucleatum strains were grown in a modified tryptone medium. Incubation for all strains was 37° C using the BBL jar Gas-Pak system. All bacterial strains after recovery from the lyophilized state were tested for purity by subculturing on TSA (BBL) with 5% sheep blood. The colonies showing typical morphology were gram stained and the organisms observed by phase microscopy. After designated growth periods in the respective media, each bacterial culture was harvested by centrifugation at 10,000 x g for 20 min and the sedimented organisms washed x 3 in 0.01 M phosphate buffer containing 0.15 M NaCl and 0.2% sodium azide (PBS), pH 7.4. The washed sedimented organisms were resuspended in PBS to a 10% suspension and kept at 4° C until used in steroid binding assays.

Buffers

All water was deonized followed by glass distillation. All pH values were determined using a Corning No. 476050 combination electrode with Beckman No. 566002 buffer as a standard, and a Corning Model 12 pH meter. TED buffer (.01 M TRIS, .015 EDTA, 5×10^{-4} DTT), TEDG buffer (TED buffer + 10% glycerol) (v/v), and TED buffer + 30% glycerol were adjusted to pH 7.4 at 0 degrees C. The percentage of glycerol in each buffer solution was checked with a Bausch and Lomb refractometer.

Cytosol Preparation

Bacteria were washed with 10% TEDG, sonicated at maximum speed, six times for 30 sec with 10 sec cooling time in between each time. The homogenate was then centrifuged at 40 K for one hour in a Beckman T50.1 Rotor. The supernatant fraction (cytosol) was carefully poured off of the pellet. The protein content of the cytosol was determined according to the method of Lowry (14) using bovine serum albumin as a standard.

Single Saturation Dose Assay

All assays were performed in duplicate. Cytosol (0.5 ml) was incubated with a final concentration of 1×10^{-8} M labeled steroid to determine total bound steroid. Cytosol (0.5 ml) was also incubated with 1×10^{-8} M labeled steroid in the presence of 100 fold excess unlabeled steroid to determine non-specific bound steroid. Each reaction mixture was then treated with dextran-coated charcoal (DCC) to remove free steroid. Dextran-coated charcoal (0.5 ml) (5 g % Norit A, 125 g % dextran in TED buffer) was pelleted by centrifugation at 2,500 g for 20 min. The reaction mixture was pipetted on to the charcoal pellet and briefly mixed with a vortex mixer. After a 10 min incubation the mixture was again centrifuged at 2,500 g for 10 min. The supernatant fluid (0.3 ml) was layered on gradients and aliquots of 0.1 ml were removed for determination of

radioactivity. Specific binding was then determined by subtracting non-specific binding from total binding.

Scatchard Analysis

For Scatchard analysis, cytosol was incubated with steroid solutions containing radioactive steroid (for 17 -estradiol ranging from 9.6×10^{-11} - 2.6×10^{-9} M for glucocorticoids using ^{3}H-dexamethasone ranging from 3.8×10^{-10} - 2.7×10^{-9} M) for 16-24 h at 0-4 degrees C. At the end of the incubation period, the total radioactivity in the samples were measured using 10 l samples. The free steroid was then extracted by the addition of 125 l of a dextran-coated charcoal suspension. (1.25 g of Norit A and .625 g dextran T-40 per 100 ml TED buffer). The charcoal-cytosol mixture was incubated for 20 min at 0-4 degrees C and the charcoal was then sedimented by centrifugation at 3,000 x g for 20 min. The radioactivity in the supernatant was then be measured using 100 l samples. The plots were corrected for nonsaturable binding.

Glycerol Density Gradient Centrifugation

Linear 10-30% glycerol gradients were prepared in 5 ml cellulose nitrate tubes using a locally constructed gradient former and a peristaltic pump (Technicon Instrument Corp., Tarrytown, New York). A portion of the cytosol (0.3 ml) was carefully layered over the gradients. The gradients were either centrifuged in a Beckman Type SW50.1 swinging bucket rotor for 16 h at 149,000 g or a Sorvall type TV865 verticle angle rotor for 2 hours at 365,000 g. Sedimentation values were determined from patterns of known standards (^{14}C BSA, ^{14}C ovalbumin, ^{14}C globulin, and catalase) run simultaneously with samples in parallel gradients. Radioactive standards were prepared by acetylation of the proteins with ^{14}C -acetic anhydride. A catalase solution was then prepared by dissolving 100 mg in 0.1 ml of TED buffer and layering 0.1 ml of the solution on the surface of the gradient. After centrifugation the absorbance of each fraction was determined at 405 nm. Fractions were collected by inserting a thin steel tube to the

bottom of the gradient and removing contents by peristaltic pump. Three drop fractions (about 0.2 mls) were collected into scintillation vials using a LKB Ultrorac fraction collector (LKB, Stockholm, Sweden). Scintillation cocktail (4 mls) was added to each tube and mixed with a vortex mixer.

RESULTS

Glycerol density gradient centrifugation profiles of cytosol obtained from Bacteroides gingivalis (CS43) were examined (Figure 14). Cytosols were incubated in 1 x 10^{-8} M (^{3}H)-dexamethasone for 22 h at 1-2° C in the presence or absence of a 100-fold excess (1 x 10^{-6} M) competing unlabelled dexamethasone or cortisol. It is evidenced by these competition studies that there exists a specific binding protein for dexamethasone in this bacteria. Concentrations of binding protein completed from the suppressible binding of (^{3}H) dexamethasone in the presence or absence of 100-fold unlabelled competition were determined (19.8 fentomoles/mg protein in 100-fold excess dexamethasone, 38.6 fentomoles/mg protein in 10-fold excess cortisol).

Similar experiments were performed with various bacteria: 10953, 10197, Fusobacterium nucleatum, XIX 9 and VI A (Table 37). No specific binding was observed in these bacteria.

Figure 14. Glycerol density gradient sedimentation patterns of Bacteroides gingivalis cytosol incubated overnight at 1-2° C with 1 x 10^{-8} (3H) dexamethasone in the presence or abscence of 100-fold excess unlabeled competing steroids. (▲) in the absence, (●) in the presence of dexamethasone, (■) in the presence of cortisol.

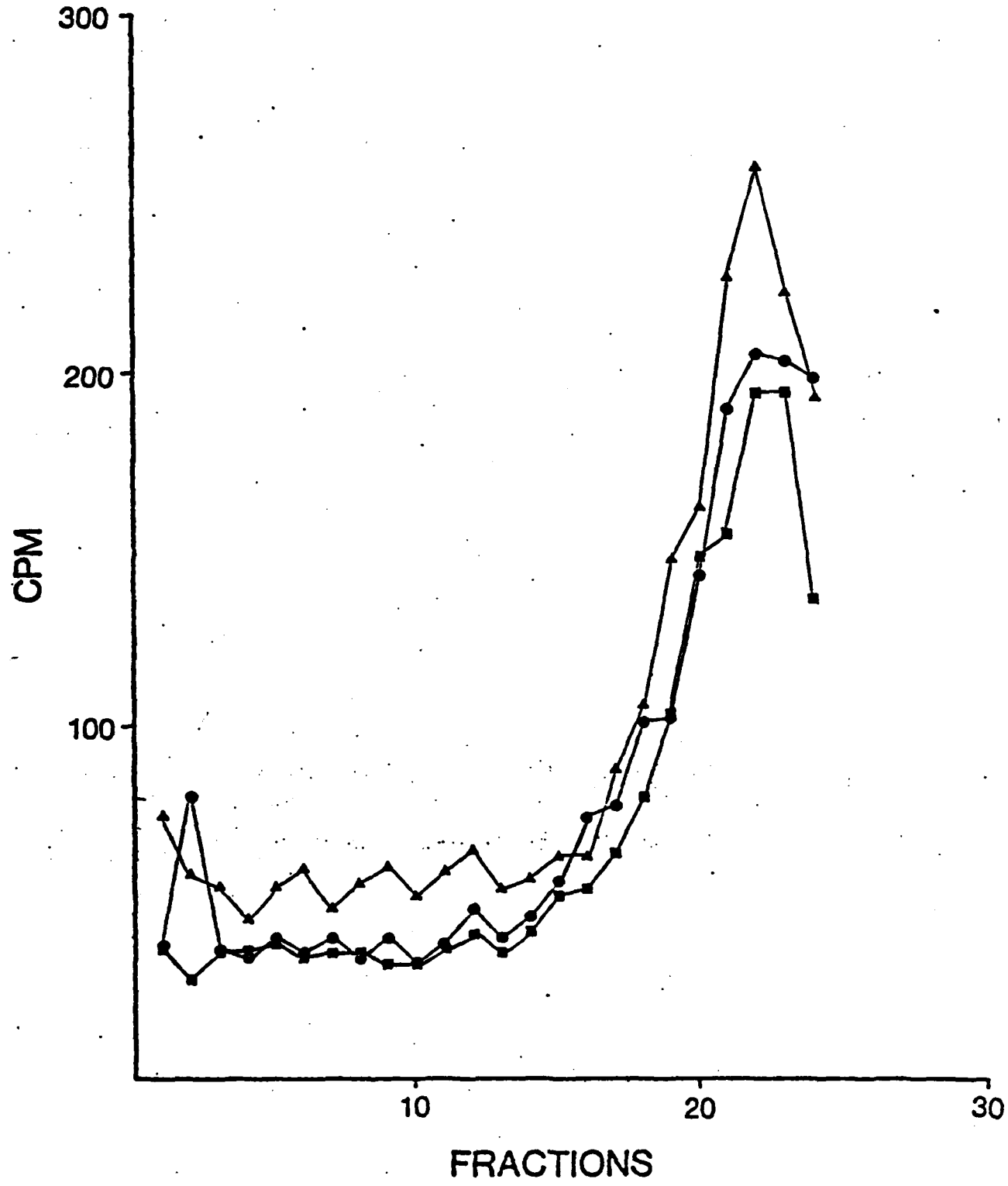

300
200
100
CPM
10
20
30
FRACTIONS

Table 37

Specifically Bound Steroid
Fentomoles/Mg Protein

	*Gluc x 10^{-6} M			*Prog x 10^{-6} M				*E_2 x 10^{-6} M	
Bacteria	DEX^{-4}	$Cort^{-4}$	$Prog^{-4}$	$Prog^{-4}$	$R5020^{-4}$	DHT^{-4}	DES^{-4}	P^{-4}	DHT^{-4}
CS43	++	++++	-	-	-	-	+	-	
10953	+	-	-						
10197	-	-			-		+		
CS44	-	-	-	-	+	-	-	-	
F. nuc.	-	-			-		+		
XIX-9 B. Int.		-	-			-		+	
XII-8 B.G.	-				-		-		
IV A Fuso.	-	-	-	-	+	-	+	-	+

DISCUSSION

Hormones are chemical signals that interact with specific target cells to promote a particular response. Steroid hormones regulate gene expression in eukaryotic cells which elects a specific response from the cell. Target cells for a particular hormone contain specialized molecules, receptors, that find the hormone and subsequently mediate its metabolic activities. A receptor has 2 roles, the first is to distinguish a particular signal from the variety of hormones and other molecules infringing on the cell, and the second is to relay this signal in such a way that the appropriate cellular response follows.

In studies with plaque flora associated with periodontal diseases, certain bacterial species are present in greater proportion than during gingival health. If periodontal disease involves increases in specific components of the indigenous population, the determination of factors which might allow or initiate this change is essential to an understanding of the etiology of the disease.

Emotional factors such as stress are associated with higher than normal levels of corticosteroids in blood serum and urine. The microorganism Bacteroides gingivalis (CS43) has been shown to possess a specific binding protein for cortisol. It is possible that the presence of higher levels of corticosteroids in the serum may allow for increased growth of these bacteria. Extensive work on the eukaryotic cell has conclusively demonstrated that specific receptors mediate metabolic processes. We have observed a high specificity glucocorticoid binding protein in these bacteria and speculate that this "receptor-like" protein is instrumental in the normal development and activity of these cells. Further studies are necessary to refine our gross characterization of this protein and qualify its functional role in the bacteria. Several variables exist in this scheme including the effect of glucocorticoid concentration on the growth of the bacteria population, rates of protein and RNA synthesis, and DNA replication. An understanding of these processes will provide an insight into the true nature of this hormone-bacteria interaction. These bacteria allow an ideal system to work with due to their high rate of turnover and general specimen availability.

Histopathological Studies

Only two of the ANUG patients allowed removal of their gingival tissue for histopathological study. The biopsy specimens (Tables 38 and 39) suggested that the type of inflammatory infiltrate was dependent upon when the biopsy was taken with respect to time after onset. As one would expect the early lesion is primarily PMNs (Table 38) and the later lesion (Table 39) shifts towards a lymphocytic infiltration.

Table 38

16 Y O B F

****Onset: 7 days prior**

ANUG 81-1498

Overall: Predominantly lymphocytes making up cellular infiltrate with PMN's confined mostly to blood vessel (capillary) areas.

Counts: 10 random fields along ulcerated area.

	Lymph's	PMN's	Mac's/Mono	Plasma	
1	49	19	0	0	
2	39	1	2	0	
3	26	2	1	10	
4	33	9	7	1	
5	20	18	1	0	
6	9	9	0	0	
7	29	12	4	0	
8	12	35	1	0	
9	81	9	0	1	
10	94	8	1	2	(1)
Total	392	122	17	14	(545)
%	72	22	3	3	

Table 39
18 Y O B F

**Onset: 3 days prior

ANUG XVI

Overall: Predominantly PMN's making up cellular infiltrate seen primarily in ulcerated regions and overlying plaque and blood. Lymphocytes also present to less extent and interspersed amng PMN's.

Counts: 10 random fields throughout ulcerated tissue areas.

	Lymph's	PMN's	Mac's/Mono	Plasma	Mast Cell
1	11	41	0	0	0
2	8	58	1	0	1
3	1	32	0	0	0
4	7	63	0	0	0
5	29	199	1	2	0
6	11	48	0	0	0
7	10	74	2	1	0
8	0	26	0	0	0
9	9	39	1	0	0
10	14	51	2	0	0
Total: (742)	100	631	7	3	1
%	13.5	85.1	0.9	0.4	0.1

BIBLIOGRAPHY

1. Vincent, H. (1896) Ann. Inst. Pasteur 10:488.
2. Orban, B. (1958) Periodontics, Mosby, St. Louis.
3. Tiecke, R. W., Stuteville, O. H. and Calandra, J. C. (1959) Pathologic Physiology of Oral Disease, Mosby, St. Louis.
4. Glickman, I. (1958) Clinical Periodontology, Saunders, Phila. and London.
5. Wilson, J. P. (1952) Am. Dent. A. J. 44:671.
6. Rosebury, T. and Foley, G. (1939) Am. Dent. A. J. 26:1800.
7. Smith, D. T. (1936) J. Infect. Dis. 46:304.
8. Kline, B. S. (1923) J. Infect. Dis. 32:481.
9. Miller, S. C. (1950) Textbook of Periodontia, Blakiston, Philadelphia.
10. Bernier, J. L. (1955) Management of Oral Disease. Mosby, St. Louis.
11. Moulton, R., Ewen, S., Thieman, W. (1952) Oral Surg., Oral Med. and Oral Path. 5:833.
12. Grupe, H. E. and Wilder, L. S. (1956) J. Periodont. 27:255.
13. Enwonwu, C. O. (1972) Arch. Oral Biol. 17:1357.
14. Goldhaber, P. and Giddon, D. B. (1964) Int. Dent. J. 14:468.
15. Wirthlin, M. R. and Devine, L. (1978) J. Periodontol. 49:449.
16. Rosebury, T. (1952) Oral Surg., Oral Med. and Oral Path. 5:363.
17. Bernier, J. L. (1944) Am. Dent. A. J. 31:1610.
18. Carter, J. J. and Bell, D. M. (1953) J. Periodont. 24:187.
19. Schulger, S. (1949) Am. Dent. A. J. 38:174.
20. Pindborg, J. J. (1951) J. Amer. Dent. Asso. 42:517.
21. Smitt, P. A. (1965) Dent. Pract. 15:281.
22. Hogan, W. J. (1951) Dent. Med. 931.
23. Zegarelli, E. V. , Kutscher, A. H. and Silvers, H. F. (1959) J. Periodont. 30:63.
24. Stout, W. C. (1957) J. Periodont. 28:314.
25. Klock, J. H. (1944) The Military Surgeon 95:43.

26. Kozol, S. M. and Schuster, H. V. (1952) Oral Surg., Oral Med. and Oral Path. 5:717.

27. Cofield, K. R., Ferguson, E. W. and Toye, A. E. (1945) Am. Dent. A. J. 32:529.

28. Hoppe, W. E. and Meyer, G. E. (1946) J. Oral. Surg. 4:304.

29. Shountoff, H. and Shountoff, W. (1954) Am. Dent. J. 48:169.

30. Alling, C. C. and Pulaski, E. J. (1959) Oral Surg., Oral Med. and Oral Path. 12:743.

31. Shinn, D. L. (1977) Excerpta Medica (S. M. Finegold, et.) pp. 334.

32. Emslie, R. D. (1967) Br. Dent. J. 122:304.

33. Duckworth, R., Waterhouse, J. P., Britton, D. E. R., Nuki, K., Sheiham, A., Winter, R and Blake, G. C. (1966) Br. Dent. J. 120:599.

34. Gilson, T. D. (1955) U. S. Armed Forces Med. J. 6:346.

35. Johnson, W. N. and Wilson, J. P. (1957).

36. Ewen, S. J. and Tascher, P. J. (1958) J. Periodont. 29:45.

37. Ewen, S. J. and Tascher, P. J. (1959) J. Periodont. 30:67.

38. Tunnicliff, R., Fink, E. B. and Hammong, C. (1936) JADA 23:1959.

39. Cahn, L. P. (1929) J. D. Res. 9:695.

40. Stammers, A. F. (1946) Brit. D. J. 81:4.

41. Schaffer, E. M. (1953) J. Periodont. Res. 24:22.

42. Listgarten, M. A. and Lewis, D. W. (1967) J. Periodont. 38:379.

43. Graykowski, E. A. and Holroyd, S. V. (1970) Dent. Clin. of N. Amer. 14:721.

44. Rosebury, T., Clark, A. R., Engel, S. G. and Tergis, F. (1950) J. Infect. Dis. 87:217.

45. Rosebury, T., Clark, A. R., Tergis, F. and Engel, S. F. (1950) J. Infect. Dis. 87:226.

46. Rosebury, T., Clark, A. R., Macdonald, J. B. and O'Connell, D. G. (1950) J. Infect. Dis. 87:234.

47. Macdonald, J. B., Sutton, R. M. and Knoll, M. L. (1954) J. Infect. Dis. 95:275.

48. Macdonald, J. B., Sutton, R. M. and Knoll, M. L. (1954) J. Infect. Dis. 95:275.

49. Hampp, E. G. and Mergenhagen, S. E. (1959) Bact. Proc. Soc. Amer. Bact. 59th General Meeting, p. 101.

50. Rizzon, H. A., Hampp, E. G. and Mergenhagen, S. E. (1961) Arch. Oral. Biol. 5:63.

51. Hampp, E. G. and Mergenhagen, S. E. (1963) J. Infect. Dis. 112:84.

52. Blake, G. C. (1968) Proc. Roy. Soc. Med. 61:131.

53. Wilton, J. M. A., Ivanyl, L. and Lehner, T. (1971) J. Periodont. Res. 6:9.

54. Harding, J., Berry, W. C. Jr., Marsh, C. and Lolliff, C. R. (1980) J. Periodontol. 51, 63.

55. Lehner, T. (1969) Br. Dent. J. 127:165.

56. Wilton, J. M. A., Ivanyi, L. and Lehner, T. (1971) J. Periodont. Res. 6:9.

57. Chung, C. P., Nesengard, R. M., Slots, J. and Genco, R. J. (1983) J. Periodontol. Sept.:557.

58. Nisengard, R. J., Myers, D., Fischman, S. and Socransky, S. (1976) J. Dent. Res. Spec. Issue B:pB206 #576, 1976.

59. Jacob, E., Allen, A. L. and Nauman, R. K. (1979) J. Clin. Micro. 10:934.

60. Jacob, E., Carter, T. B. and Nauman, R. K. (1980) J. Clin. Micro. 12:610.

61. Slots, J. and Zambon, J. J. (1982) R. J. Genco and S. E. Mergenhagen (eds.), Host-Parasite Interactions in Periodontal Diseases, p. 27. Washington, D. C., American Society for Microbiology, 1982.

62. Loesche, W. J., Syed, S. S., Laughon, B. E. and Stoll, J. (1981) J. Periodontol. 53:223.

63. Spiegel, C. A., Hayduk, S. E., Minah, G. E. and Krywolap, G. N. (1979) J. Perio. Res. 14:376.

64. Falkler, Jr., W. A. and Hawley, C. E. (1976) Infect. and Immun. 15:230.

65. Falkler, Jr., W. A., Smoot, C. N. and Mongiello, J. R. (1982) Archs. oral Biol. 27L553.

66. Crawford, Y. E., Nalewalk, R. P., Lythe, R. I. and O'Connell, J. L. (1971) Infect. and Immun. 4:212.

67. Holdeman, L. F. and Moore, W. E. C. (1974) p. 384-418. In Bergeys Manual of Determinative Bacteriology, Eighth Edition. E. Buchanan and N. E. Gibbons, Editors. The Williams and Wilkins Company, Baltimore, Md.

68. Falkler, W. A. Jr. and Burger, B. W. (1981) Archs oral Biol. 26:1015.

69. Weir, R. M. (1969) p. 367-368. Handbook of Experimental Immunology. F. A. Davis Co., Philadelphia, PA.

70. Garcia, M. M., Charlton, K. M. and McKay, K. A. (1975) Infect. Immun. 11:371.

71. Hase, S., Hofstad, T. and Rietschel, E. T. (1977) J. Bacteriol. 129:9.

72. Okuda, K. and Takazoe, I. (1974) Archs oral Biol. 19:415.

73. Kondo, W., Sato, M. and Ozawa, H. (1976) Archs oral Biol. 21:363.

74. Slots, J. and Gibbons, R. J. (1978) Infect. Immun. 19:254.

75. Mongiello, J. R. and Falkler, W. A. Jr. (1979) Archs oral Biol. 24:539.

76. Nowotny, A. (1963) J. Bacteriol. 85:427.

77. Hofstad, T. and Sveen, K. (1979) Scand. J. of infect. Dis. (Suppl. 19:42.

78. Ribi, E., Haskins, W. T., Milner, K. C., Anacker, R. L., Ritter, D. B., Goode, G., Trapani, R. J. and Landy, M. (1962) J. Bacteriol. 84:803.

79. Hofstad, T. (1969) J. Bacteriol. 97:1078.

80. Kristoffersen, T. 1969. Immunochemical studies oforal Fusobacterium 2. Some properties of undigested cell wall prearations. Acta path. microbiol. Scand. 77:247-257.

81. Hofstad, T. (1974) J of Gen. Microbiol. 85:314.

82. Sveen, K. (1977) The Capacity of lipopolysaccharides from Bacteroides, Fusobacterium, and Veillonella to produce skin inflammation and the localized Shwartzman reaction in rabbits. J. of Periodont. Res. 12:340-350.

83. Hofstad, T. 1968) Archs oral Biol. 13:149.

84. Vasstrand, E. N., Hofstad, T., Endersen, C. and Jensen, H. B. (1979) Infect. Immun. 25:775.

85. Walker, C. B., Ratliff, D., Muller, D., Mandell, R. and Socransky, S. J. (1979) J. CLin. Microbiol. 10:844.

86. Rose, N. R. and Friedman, H. (1980) Manual of Clin. Micro. (2nd ed.) Amer. Soc. for Microbiol., Washington, D. C.

87. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193:265.

88. Poxton, I. R., (1979) J. Clin. Path. 32:294.

89. Darwish, S., Hyppa, T. and Socransky, S. S. (1978) J. Periodontal. Res. 13:1.

90. Slots, J. (1977) Scand. J. Dent. Res. 85:114.

91. Kornman, K. S., Holt, S. C. and Robertson, P. B. (1981) J. Periodontal. Res. 16:363.

92. Slots, J. (1977) Scand. J. Dent. Res. 85:114.

93. Syed, S. A., Svanberg, M. and Svanberg, G. (1981) J. Clin. Periodont. 8:45.

94. Tanner, A. C. R., Haffer, C., Bratthall, G. T., Visconti, R. A. and Socransky, S. S. (1979) J. clin. Periodont. 6:278.

95. Falkler, W. A., Jr., Smoot, C. N. and Mongiello, J. R. (1982) Arch. oral Biol. 27:553.

96. Slots, J. and potts,T. (1982) Int. J. Syst. Bacteriol. 32:191.

97. Adams, R. A., Zander, H. A. and Polson, A. M. (1979) J. Periodont. 50:7.

98. Kennedy, J. E. and Polson, A. M. (1973) J. Periodont. 44:140.

99. Matsson, L. and Attstrom, R. (1979) J. Clin. Periodont. 6:186.

100. Mouton, C., P. Hammond, J. Slots and R. J. Genco (1980) J. Clin. Microbiol. 11:682.

101. Reed, M., J. Slots, C. Mouton and R. J. Genco (1980) Infect. Immun. 29:564.

102. Abshire, R. L., V. R. Dowell and G. L. Lombard (1979) J. Clin. Microbiol. 9:274.

103. Elhag, K. M., K. A. Bettelheim and S. Tabaqchali (1977) J. Hyg. 79:233.

104. Hsu, P. C., B. H. Minshew, B. L. Williams and E. S. Lennard (1979) J. CLin. Microbiol. 10:285.

105. Kasper, D. L. (1976) J. Infect. Dis. 1337:79.

106. Kasper, D. L., A. P. Fiddian and S. Tabaqchali (1979) Lancet 1 (8110):239.

107. Kasper, D. L., M. E. Hayes, B. G. Reinap, F. O. Craft, A. B. Onderdonk and F. B. Polk (1977) J. Infect. Dis. 136:75.

108. Reinhold, L. (1971) Hyg. 216:219.

109. Cherniak, R., G. L. Lombard and V. R. Dowell (1979) Immunochemical evidence for multiple serotypes of Bacteroides fragilis. J. Clin. Microbiol. 9:699.

110. Elhag, K. M. and S. Tabaqchali (1978) J. Hyg. 80:439.

111. Abshire, R. L., G. L. Lombard and V. R. Dowell (1977) J. Clin. Microbiol. 6:425.

112. Babb, J. L. and C. S. Cumminns (1981) J. Clin. Microbiol. 13:369.

113. Bideberry, D., D. Leage, J. C. Petit and G. L. Daguet. (1980) La Nouvelle Presse Medicale 9:2080.

114. Landay, M. E. and W. Baldwin (1979) J. Clin. Microbiol. 9:643.

115. Rissing, J. P., J. G. Crowder, J. W. Smith and A. White (1974) J. Infect. Dis. 130:70.

116. Stauffer, L. R., E. O. Hill, J. W. Holland and W. A. Altemeier (1975) J. clin. Microbiol. 2:337.

117. Weintraub, A., A. A. Lindberg and C. E.Nord (1979) Med. Microbiol. Immunol. 167:223.

118. DeGirolami, P. C. and Chandrakant, p. M. (1981) Amer. J. of Clin. Path. 76:78.

119. Hofstad, T. (1977) Acta. Pathol. Microbiol. Scand. Sect. B 85:9.

120. Marx, A., M. Petcovici, J. Bittner, M. Antohi andJ. Ardeleanu (1982) Infect. Immun. 36:943.

121. Poxton, I. R., R. Brown and J. G. Collee (1982) J. Med. Microbiol. 15:223.

122. Leschine, S. R. and Canale-Parola (1980) J. Clin. Microbiol. 12:792.

123. Gochenour, W. A., Yager, R. M., Wetmore, P. E. and Hightower, J. A. (1953) Am. J. Pub. Hlth. 43:405.

124. Swindlehurst, C. A., Shah, H. N., Parr, C. W. and Williams, R. A. D. (1977) J. Appld. Bacteriol. 43:319.

125. Payne, J. W. (1976) Chromatographic and Electrophoretic Techniques (Vol. III). Zonal Electrophoresis, Yearbook Medical Publishers, Inc., Chicago, p. 321.

126. Masuda, K. and Kawata, T. (1982) J. Bacteriol. 15:1405.

PAPERS AND ABSTRACTS RESULTING FROM THIS STUDY

Papers

J. W. Vincent, W. A. Falkler, Jr. and J. A. Craig. Comparison of Serologic Reactions of Typed Fusobacterium nucleatum Strains with Isolates from Humans, Canines and a Macaca mulatta Monkey. J. Clin. Micro. 17:631-635, 1983.

W. A. Falkler, Jr., E. B. Clayman and D. F. Shaefer. Hemolysis of Human Erythrocytes by the Fusobacterium nucleatum Found in Periodontal Diseases. Archs. oral Biol, 28:735-739, 1983.

D. F. Shaefer and W. A. Falkler, Jr. Reaction of Clinical Isolates and Typed Strains of the Family Bacteroidaceae with Rabbit Anti-Bacteroides Serum by Radial Immunodiffusion. J. Clin. Micro. 18:18-22, 1983.

J. B. Suzuki, W. A. Falkler, Jr. and J. W. Vincent. Lifestyle and Clinical Evaluation of Metropolitan ANUG Patients. in preparation.

W. A. Falkler, Jr., S. A. Martin, J. B. Suzuki and J. W. Vincent. Bacteriological Study of ANUG Lesions. in preparation.

Abstracts

Extracellular Hemagglutinating Antigens from Broth Cultures of Bacteroides gingivalis. Shaefer, D. F. and W. A. Falkler, Jr. Abstr. Ann. Mtg. ASM, 1982

Serologic Studies of Fusobacterium nucleatum from Different Oral Lesions. W. A. Falkler, Jr., J. W. Vincent, R. Lai and J. B. Suzuki, 1982 IADR Meeting

Hemolysis of Human Cells by Fusobacterium nucleatum. E. B. Clayman, D. F. Shaefer and W. A. Falkler, Jr. Abstr. Ann. Mtg. ASM, 1981.

Human Precipitating Antibody Reactive with Eubacterium brachy. J. W. Vincent and W. A. Falkler, Jr., 1983 AADR Meeting.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoretic Analysis of oral Spirochetes. B. D. Tall and R. K. Nauman, 1983 ASM Meeting.

Corticosteroid Receptors in Bacteroides gingivalis. W. A. Falkler, Jr., M. Salah and N. Bashirelahi, 1983 IADR Meeting.

Serologic Reactions of F. periodonticum. M. Romagnoli, W. A. Falkler, Jr., J. B. Suzuki and J. W. Vincent, submitted to 1985 IADR meeting.

Graduate Students Supported by this Contract

Rose Lai	received M. S. degree 12/20/82
David Shaefer	Ph.D. candidate
Susan Mandel	M. S. candidate
John Czeculin	M. S. candidate
Stephen Martin	Ph.D. candidate
Mark Romagnoli	M. S. candidate
Michael Salah	received D.D.S. degree 6/1/84
Eric R. Hall	M. S. candidate
Joy R. Miller	received M. S. degree 1/5/84
Ben Tall	Ph.D. candidate

DISTRIBUTION LIST

4 copies: Commander
U. S. Army Medical Research and Development Command
ATTN: SGRD-SI
Fort Detrick
Frederick, MD 21701

12 copies: Administrator
Defense Technical Information Center
ATTN: DTIC-DDA
Cameron Station
Alexandria, VA 22314

1 copy: Superintendent
Academy of Health Sciences, US Army
ATTN: AHS-COM
Fort Sam Houston, TX 78234

1 copy: Dean, School of Medicine
Uniformed Services University of the Health Sciences
4301 Jones Bridge Road
Bethesda, MD 20014

END

10-86

DTIC